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Vol. LVIII, No. 1 FEBRUARY, 1930

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY
Woods Hole, Massachusetts

THE FIRST REYNOLD A. SPAETH MEMORIAL LECTURE[1]

The Present Conception of the Structure of the Plasma Membrane

RUDOLPH HÖBER

Physiologisches Institut, Kiel

Ladies and gentlemen: I feel in this moment that more than ever since the beginning of my scientific life, I have sympathies with this country, where the modern view of general physiology, to which I myself have devoted my life's work, has been developed with perhaps greater success than anywhere else. More than ever here in Woods Hole I feel the genius of Jacques Loeb, who, as no one else since the days of Claude Bernard, taught us so impressively that the most important task of physiology lies in recognizing the general properties of living matter, and who spent here in this place the happiest days of his life doing research work. And sadly here too I remember at this hour my friend, Reynold Spaeth, in whose memory I have the honour to give you this lecture today. Sixteen years ago he came to Kiel with his young wife, an enthusiastic young scientist, eagerly longing to take up physical chemistry as his weapon with which to advance into the undiscovered land of science. And then after his return from Germany, like his great idol Jacques Loeb and many of Loeb's students, he learned to love above all the scientific atmosphere of Woods Hole, —he who was destined to leave us so early, disregarding in his intense eagerness for research the dangers of the tropics.

The *genius loci* of Woods Hole, who apparently holds his protecting hand over general physiology with particular kindness, also moves me to take as the theme of this lecture the present conception of the structure of the plasma membrane. I am sure that with this theme

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[1] Delivered at the Marine Biological Laboratory, Woods Hole, on September 9, 1929. The announcement of the foundation of the Spaeth Memorial Lecture will be found in the Report of the Director of the Laboratory for 1928 (*Biol. Bull.*, 1929, **57**: 22).

I shall enter the sphere of interest of many people who have performed and are still performing physiological studies at Woods Hole. At once, with this theme I recall to mind the investigations which Reynold Spaeth put forward with so much skill, perseverance and enthusiasm during his short residence in my laboratory.

Ladies and gentlemen, many of you will agree with me that the problem of cell permeability belongs among the most urgent questions of general physiology, I daresay perhaps of special physiology too. For the working out of a theory of permeability is intimately joined with the understanding of many fundamental phenomena of life, such as nutrition, secretion, absorption, excretion, growth and irritability. Hence let us begin to follow a little the development of the doctrines of the permeability of cells.

In 1855 Naegeli described the phenomenon of plasmolysis of the plant cell, consisting in a persisting retraction of the protoplast from the cell wall, if the cell is bathed in what we call today a hypertonic solution. Pfeffer in 1877 gave an explanation of the permanent plasmolysis, comparing the plant cell with the "Traubesche Zelle" made up by a precipitation membrane, for instance, by a copper ferrocyanide membrane. He suggested that the protoplast is surrounded by some limiting layer on the outer surface, the plasma membrane having, like the inorganic precipitation membrane, the property of semipermeability, that is, permeability to water but impermeability to such dissolved substances as produce the plasmolysis. Some time later, Overton gave an unquestionable proof that Pfeffer's assumption was correct; making use of a series of organic compounds, he showed that, in full harmony with the theory of solutions based by van't Hoff upon the experiments of Pfeffer, all solutions which produce the beginning of plasmolysis have the same molecular concentration. Thus at first one was compelled to assume that the interior of the living cell was shut off from dissolved substances and that only water was able to enter.

The next important step in recognizing the nature of the limiting membrane of the cell was the discovery of Klebs and de Vries that besides the dissolved substances which cause permanent plasmolysis, there exist some others,—for instance glycerol and urea, which given in hypertonic solution, instead of bringing about the permanent plasmolysis, only produce the initiation of shrinking, which is followed sooner or later by deplasmolysis. Furthermore, Overton and others have discovered a great many substances that do not plasmolyse at all. It was only a logical outcome of the theory of the plasma membrane to explain all this by the assumption that for such substances the membrane is not impermeable, but allows them to pass faster or slower. These conclusions could be often established beyond doubt by chemical, optical and other forms of analysis of the contents of the cells.

But the question now arises as to whether the inorganic precipitation membranes behave in perhaps the same manner, that is, whether or not they are permeable to the same substances to which the plant cells, as indicated by the plasmolysis experiments, are permeable. Curiously enough, this question, which is derived so easily from the experiments, has been answered only recently by the systematic experiments of Collander.[2] This author showed that the copper ferrocyanide membrane behaves also in a very different manner in relation to a great number of organic non-electrolytes, allowing some to pass not at all, others to pass slowly, and still others quickly. But the laws governing the speed of permeation through the precipitation membrane differ widely from those which hold good in the case of the plasma membrane, as is illustrated by Table I.

TABLE I

Substance	Relative permeability of *Rhœo discolor*	Permeability of Copper Ferrocyanide	Molecular Volume	Relative Solubility in Ether
Methyl alcohol	125	+++++	8.2	0.273
Ethyl alcohol	71	++++	12.8	1.86
Valeramide	69	++	28.7	0.170
Ethyl urethane	59			0.637
Ethylene glycol	4.4	++++	14.4	0.0068
Diethylurea	2.0			0.0185
Glycerol	1.3	+++	20.6	0.0012
Methylurea	1.2			0.0012
Urea	1.1	+++++	13.7	0.0005
Glucose	1.02	+	37.5	<0.0001
Glycocoll	1.0	++++	17.1	<0.0001
Saccharose	1.0	+	70.4	<0.0001

The table contains data with respect to the behavior of twelve organic non-electrolytes. The first series of numbers shows the various speeds of permeation as related to the epidermis cells of *Rhœo discolor*, the second the speeds of permeation in relation to the copper ferrocyanide membrane. It can be easily seen that between both there exists no parallelism at all. The third series indicates the molecular volumes calculated by Collander from the values of molecular refraction; the fourth series gives their relative solubilities in ether. Now comparing the second and third series, we recognize clearly that the velocity of permeation of the precipitation membrane is a function of molecular volume. This governing rule being established, the character of the membrane is immediately revealed. It behaves as a sieve for molecules so that the size of its pores determines whether or not the dissolved substance can permeate. Such a membrane is semipermeable as soon as the diameter of the molecules of the solution surpasses a certain size. The passage is then allowed only to water, for its molecules are characterized by an especially small volume. And since it is highly probable that the pores of the membrane are not all of the same size, the molecules with a diameter below the limiting value have a greater chance to slip through, as they are smaller. Furthermore, the fourth series of numbers shows that the permeability of the plasma membrane might depend upon quite another principle, that is, the principle of solubility in the substance of which the membrane is composed or, briefly, the principle of selective solubility. Thus we come to speak of the first comprehensive theory of cell permeation, the lipoid theory of Overton.

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[2] Collander, *Kolloidchem. Beihefte*, **19**, 72, 1924 and **20**, 273, 1925.

It is a well-known fact that the lipoid theory has been supported by a large amount of powerful arguments, but it is also well-known that one has struggled sharply against it, very often, I believe, with insufficient arguments. But even today the theory cannot conclusively be judged for the simple reason that the physico-chemical foundation is partly too narrow and partly too uncertain. Professor Jacobs was indeed completely right when he wrote a short time ago: "It may be emphasized that what is most needed in the field of cell permeability at the present day is facts." As everybody knows, Overton based his theory in the first place on the comparison between the speed of penetration of substances and their relative solubility in oil. Collander, recently reviewing most carefully the experiments of Overton on plant cells, advocated especially the correspondence between permeability and solubility in ether.[3] Both these authors are quite clear about the limited value of their comparison, and the table also shows that the parallelism is fairly incomplete. Therefore the lipoid theory is still nowadays a *petitio principii*. However, the thesis that the permeability to the organic non-electrolytes is to be compared to the solubility in organic solvents agrees so often with the experimental data, that I myself have practically no doubt that it is only necessary to discover such solvents as might be still better suited to comparison with the material of the plasma membrane than oil or ether. It is really astonishing that since the lipoid theory was set up more than thirty years ago, so little systematic research work has been done on the relative solubility of organic compounds in different organic solvents comparable to the lipoids of Overton, in order to get a firm basis for the theory. It is well known that an interesting attempt to find a better model was made by Nirenstein some years ago.[4] He showed that several exceptions to the rule previously given by Paul Ehrlich, that the vital colors which enter easily into the living cell dissolve in oil, could be removed by trying to imitate the plasma membrane with a mixture of an oil with a fatty acid and an organic fat-soluble base. Table II shows experiments by which I was able to compare the relative solubility of acid dyes in the above-mentioned oil mixture with the relative absorption of colors by red blood corpuscles.[5]

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[3] Collander and Bärlund, *Soc. scient. fenn.*, **2**, 9, 1926; Bärlund, *Acta botan. fenn.*, **5**, 1929.

Table II

Sulfonic Acid Dyes	Relative Solubility in Oil Mixture	Relative Absorption by Blood Corpuscles
Wollgrun, Lichtgrun Cyanol, Eriocyanin	0	0
Azofuchsin I	< 1	0.3–1
Azofuchsin G	< 1	<1
Bromophenol blue	27	1–3
Tropæolin 1	30	1–3
Tropæolin 2	30	4–8
Orange R	85	7–16
Brilliant orange R	71	7–16
Metanil yellow	94	10–16

It can be seen that there exists a parallelism between solubility and absorption, and it is especially noteworthy that this similarity means not only intensity of staining, but means permeability; for it follows from the table that dyes which are not dissolved in the oil mixture do not enter the blood corpuscles at all. However, such experiments with dyestuffs do not come out quite satisfactorily, as I can easily show. Therefore it is necessary to collect further experimental data to get a clear understanding, inasmuch as the cell permeability is a solution permeability.

But there can be no doubt that the cell permeability is not only a solution permeability with regard to an oil-like solvent within the plasma membrane. In the first place in this connection it is a very striking fact that water enters the cell usually with remarkable speed, because it is impossible to reconcile this entrance with the supposition that the membrane consists entirely of an oily phase. Secondly there exists an apparent permeability of certain kinds of cells to inorganic anions, though the inorganic salts are generally not in the least soluble in organic solvents. Thirdly, there are important nutritive materials which cannot get into the cell in any way, but belong also to those substances that are nearly or entirely insoluble in the organic solvents.

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[4] Nirenstein, *Pflüger's Arch.*, 179, 233, 1920.

[5] Unpublished experiments.

Now we are able to interpret the first and second points by returning to the already-mentioned sieve theory of permeability of the precipitation membranes, and we will see that on that account the comprehension of the structure of the plasma membrane receives a very important supplement. More than thirty years ago Koeppe, Gürber and Hamburger made the discovery,—which has often been verified since,—that the red blood corpuscles have a selective permeability for anions. It is well known that this property has the greatest importance for the buffer capacity of the blood; but it seemed for a long time to be a strange *unicum*, for which there existed hardly any physical parallel. Otherwise it might have been possible to construct a model to imitate the peculiarities of the membrane of the blood corpuscles. Today the matter is practically clear; the well-known experiments of Michaelis and Collander with artificial membranes, especially with dried collodion membranes, enabled us to understand the singular phenomenon. Michaelis proved that these membranes are, under certain circumstances, the seat of a great potential difference, whose direction and amount is an obvious sign that the membranes are exclusively cation-permeable.[6] Therefore there is an analogy between the cation-permeable collodion membrane and the anion-permeable blood corpuscle membrane. At the collodion membrane the anion plays no rôle, whereas a cation gives rise to an electromotive force which increases as its migration velocity increases or as its diameter decreases. On account of these facts Michaelis has proposed the following hypothesis: the membrane allows only the cation to pass through it as through a sieve; the ions with the smallest diameter pass with the greatest speed, and the entrance of ions into the pores of the membrane is prevented if their diameter exceeds a certain value. That is apparently the reason why, for instance, the earth-alkali ions are unable to pass some collodion membranes characterized by rather narrow pores. In this way we may understand that a quantity of an ion sufficient to be detected by chemical methods can penetrate only if there is present another cation on the other side of the membrane, so that an exchange can take place. That is exactly the same as with the red blood corpuscles, where from the beginning the demonstration of the selective anion-permeability depended upon the fact that as long as there exist differences of concentration in the proper direction, the anions of the surrounding solution can be exchanged against the anions of the interior of the cell.

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[6] Michaelis, *Naturwissenschaften* 1926, **14**: 33.

And now the question arises, how it is to be understood, that in the case of the collodion membrane the pore permeability is limited to the cations and, in the case of the red blood corpuscles, to the anions. Michaelis had already turned his mind to the fact that the substance of the cation-permeable membrane itself is negatively charged, and he connected this idea with the well-known membrane studies of Bethe and Toropoff [7] and the experiments on the reversal of membrane potentials in gelatine discs, which have been established by Matsuo in my own laboratory.[8] As a matter of fact it can be proved that this idea is right. There exists a relation between the electric charge of the membrane material and the faculty of the ions with opposite electric charge to pass. In my laboratory Mond succeeded in demonstrating that if the negative charge of the collodion is changed to a positive charge by addition of a basic dye, for instance by rhodamin, the membrane thus formed, instead of being exclusively cation-permeable is changed into a membrane of selective anion-permeability.[9] Table III illustrates the resulting conditions.

TABLE III

Membrane Potentials in Rhodamin-collodion Membranes

	– – – → Cl			
0.1M NaCl	←―――――――――――― – – – →	0.1M NaSCN	+ 60	millivolts
0.1M NaCl	←―――――――――― – – – →	0.1M NaI	+ 33	"
0.1M NaCl	←―――――― – – – →	0.1M NaBr	+ 20	"
0.1M NaCl		0.1M NaCl	0	"
0.1M NaCl	←―― – – – →	0.1M Na_2SO_4	− 3.8	"
0.1M NaCl		0.1M KCl	+ 2	"

$SCN > I > Br > Cl > SO_4$

Cations without effect

The dotted arrows show the direction of the movement of the chlorine ions; their length is a measure of the potential dependent on the velocity of the ions. The arrows drawn refer in a corresponding manner to the anions of the opposite side of the membrane. The electromotive forces decrease from + 60 millivolts to − 3.8 millivolts along the series of anions: thiocyanate, iodine, bromine, chlorine, sulfate. The cations are without any effect. The membrane potential is therefore approximately zero if there is sodium chloride and

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[7] Bethe and Toropoff, *Zeitschr. f. physik. Chemie*, **88**, 686, 1914 and **89**, 597, 1915.

[8] Matsuo, *Pflüger's Arch.*, **200**, 232, 1923.

[9] Mond and Hoffmann, *Pflüger's Arch.*, **220**, 194, 1928.

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potassium chloride in the same concentration on each side of the membrane.

There can be no doubt that these experiments demonstrate on the one hand in a very conclusive manner the existence of anion-permeability on a membrane originally cation-permeable, but they reveal on the other hand some difficulties in our understanding of these and, as we shall see, of other alterations of the ion-permeable membranes. The membrane potential of the rhodamin collodion membrane does not increase with increasing migration velocity of the effective ion, as has been found by Michaelis with the cation-permeable collodion membrane, but the potential changes according to the lyotropic series. This seems to point to some kind of relation of ion-permeability to the colloidal state of the membrane, which is known to depend in an especially characteristic manner on the lyotropic properties of the ions.

However, before discussing this question more amply, we will look at a remarkable consequence of the membrane experiments just described. Mond, supposing that the membrane material of the red blood corpuscles is electropositive, suggested that their natural anion-permeability might be turned into cation-permeability, if one succeeds in giving the membrane substance a negative charge.[10] This actually happens by the addition of a suitable amount of hydroxyl ions. As soon as the reaction in the surrounding medium of the blood corpuscles is made more alkaline than pH 8, the usual selective anion-permeability is displaced by selective cation-permeability, so that now an exchange between the potassium ions of the interior with the sodium ions of the environmental solution begins, while the chlorine and bicarbonate ions present in both serum and corpuscles, which were up to this point able to pass through, are now fixed. Mond has advocated the view that the decisive constituent of the plasma membrane, to which the opposite charge is to be attributed, has ampholyte character and might be globine, that is, a protein body, because the reaction by which this reversal of anion-permeability into cation-permeability takes place conforms with the isoelectric point of the globine, which is pH 8.1.

In this way we come to a conception, similar to the well-known hypothesis of Nathansohn, that the cell surface is comparable to a mosaic of both lipoids and proteins. Apparently the plasma membrane of the red blood corpuscles consists of at least two constituents, a lipoid phase, whose existence enables the lipoid-soluble substances to enter, and a protein phase, which is pore-permeable, so that water as well as dissolved substances, whose molecular size is small enough, can pass through. As to the character of the structure of the cation-permeable membranes, which we will now discuss, the opinion is not yet substantiated enough.

---

[10] Mond, *Pflüger's Arch.*, **217**, 618, 1927.

TABLE IV

Resting Potentials of the Sciatic Nerve

Time	Potential	Solution	Time	Potential	Solution
	millivolts			*millivolts*	
3:21	20.6	Ringer	3:20	27.4	Ringer
3:53	20.5	Ringer	3:52	27.5	Ringer
3:55		Ringer with 0.08% KCl	3:54		Ringer with 0.08% KCl + 0.1% $CaCl_2$
4:03	19.0		4:02	27.7	"
4:28	17.2		4:27	28.5	"
5:18	16.5		5:17	28.6	"

It seems that the cation-permeable membranes exist more frequently than anion-permeable membranes. As Bernstein and I have pointed out twenty-five years ago, the hypothesis of selective cation-permeability gives a good explanation of the electro-negativity that results from injury and of negativity resulting from activity. But if we produce a difference of potential in uninjured tissues such as muscles, liver and apple by joining their surfaces at two different points with two different salt solutions, then we see that, although the cations are better enabled to produce an electric current (apparently in connection with the negative character of the membrane colloids), the anions have an action too, the strength of which is correlated with their position in the lyotropic series: sulfate, chlorine, bromine, nitrate, iodine, thiocyanate. But the cations also do not act exactly according to the series of the size of the ions: cæsium, rubidium, potassium, sodium, lithium, but succeed each other as potassium, rubidium, cæsium, sodium, lithium,—a series met with rather often, as I have found in relation to changing the hydrophilic colloidal state, and characterized by the peculiar dislocation of cæsium. And finally, regarding the membrane potentials of muscle and nerve, we encounter a cation antagonism, for example that between potassium and calcium, which might be explained by assuming an influence on the state of membrane colloids, whereas it is difficult to explain it by supposing the existence of a sieve-like membrane, the size of whose pores remains unchangeable. Table IV gives as an example the behavior of the sciatic nerve of a frog.[11]

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[11] Höber and Strohe, *Pflüger's Arch.*, **222,** 71, 1929.

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As the experiment on the left side demonstrates, the resting potential falls if the uninjured surface of the nerve is brought into contact with a Ringer's solution in which the percentage of potassium chloride is raised to more than 0.08. If we increase not only the concentration of potassium chloride but also of calcium chloride to 0.01, the alteration of the initial potential, as is to be seen in the experiment on the right side of the figure, does not take place. So we notice again in regard to the membrane potentials the well-known antagonism between potassium and calcium, and since there is hardly any doubt that the permeability of the plasma membrane due to its porous structure plays a significant rôle, we concluded that this permeability is, according to the nature of the composing material, much more variable than the pore-permeability of the artificial ion-permeable membranes, especially of the collodion membranes.

Before leaving the interesting question of ion-permeability, I wish to direct your attention to a membrane with very curious qualities. Last year I set up and examined a membrane which was a patchwork of cation-permeable pieces of collodion and anion-permeable pieces of rhodamin collodion.[12] Figure 1 gives the scheme. This membrane must have the following qualities, and in fact it does have them. If we place a salt solution on one side of it, for instance, a solution of potassium chloride, and on the other side water, the salt cannot diffuse into the water, although the membrane is as permeable for the potassium ions as for the chlorine ions, because a passage in chemically detectable quantity would be possible only if it could happen at just the same place in equivalent amounts of cation and anion, or in other words, because one ion can move only at an infinitesimal distance from the opposite. However, the passage of the potassium chloride is rendered possible as soon as a salt, whose ions can interchange through the membrane with the potassium and the chlorine ions, is placed on the other side of the membrane. It seems to me that membranes of this kind, which, in spite of their permeability for anion and cation, are able to entirely prevent the escape of salts, have been realized by nature and play an important rôle.

Now the question arises as to whether, in addition to the water, only inorganic ions take the way through the pores of the plasma membrane. Logically the answer is no. For if there are molecules whose volume is of the same order as that of the permeating ions, then they naturally must take the way through the pores, regardless of the possibility of their passing equally well through the membrane by selective solution. Of course it has been pointed out by Michaelis that the collodion membrane, if it is dried enough to establish selective ion-permeability and therefore to give the maximum electromotive effect, allows those molecules to pass whose diameter is about the same as that of glucose.[13] A similar behavior is met with in the plasma membranes. It will be noted that among the organic non-electrolytes entering into the cell, there are some which permeate more quickly than might be expected in relation to their relative lipoid-solubility, or, more correctly, in respect to their relative solubility in ether, supposing that the relative solubility in ether is to be acknowledged as a likely measure of the physiological phenomenon. Some of these substances are characterized by a relatively small molecular volume, for instance, ethylene glycol and glycerol. Therefore Collander may be quite right in considering their comparatively rapid permeation into plant cells as due to the porosity of the plasma membrane.[14] In other cases where a disagreement occurs between velocity of penetration and solubility in ether, for example with urea and its derivatives, even the view of a sieve-like property fails to overcome the difficulties. But here we can see, as I have found with Watzadse, that the difficulties will be removed if, instead of the solubility in ether, the solubility in the previously mentioned oil mixture of Nirenstein will be correlated with the physiological phenomenon.[15]

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[12] Höber and Hoffmann, *Pflüger's Arch.*, **220**, 558, 1928.

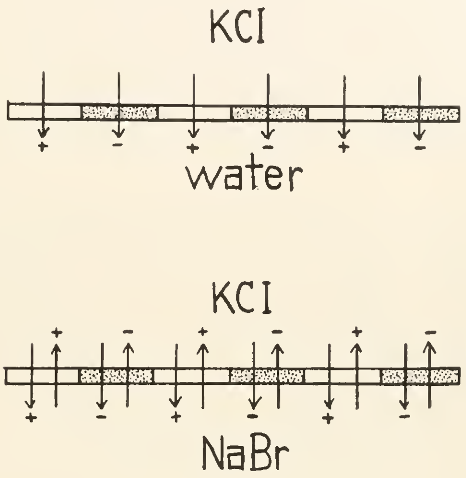

FIG. 1.

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[13] *Loc. cit.*

[14] *Loc. cit.*

[15] Watzadse, *Pflüger's Arch.*, **222**, 640, 1929.

The assumption of the porosity of the plasma membrane in this manner being justified in several ways, it will be necessary to study as intimately as possible the properties of the artificial porous membranes and especially, because of their great stability, those of dried collodion membranes. Therefore perhaps it is not too audacious to consider the possibility, in relation to physiological conditions, that certain molecules with a diameter not too great and not too small might be stopped in the pores and obstruct them in the same manner that ultramicroscopic particles are not only kept back by an ultrafilter, but finally also obstruct its pores.

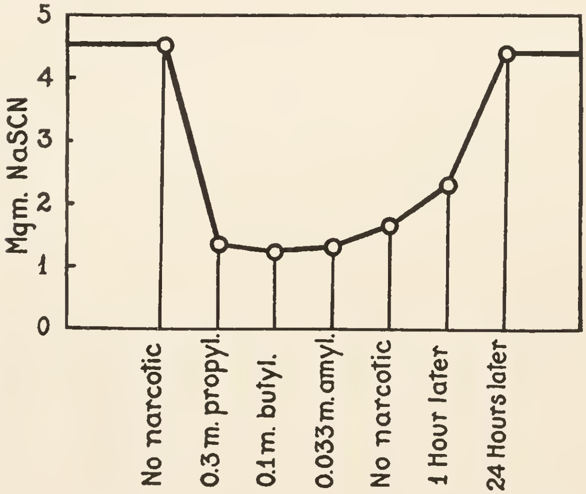

FIG. 2. Diffusion of thiocyanate in 15′, retarded by urethanes.

From this point of view Anselmino has made experiments in my laboratory. He favored the obstruction of the pores by using narcotics, because they can be adsorbed by the collodion.[16] The result was that the collodion membrane was obstructed to such a degree that the osmotic movement of water as well as the diffusion of molecules of small size was strongly retarded. Figure 2 reproduces a striking experiment. You see that the diffusion of thiocyanate is reversibly slowed by several urethanes, and that the homologous urethanes exert their influence characteristically so that the longer their carbon chains are, the smaller their limiting concentration will be, in the same way that we usually observe in narcosis.

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[16] Anselmino, *Pflüger's Arch.*, **220**, 524, 1928.

It will be necessary to find out still more exactly which substances are suitable for the obstruction of the pores and which are not. Michaelis has recently found that the speed of diffusion of glucose through a collodion membrane of suitable pore size decreases with time more and more, and he regards this too as effectuated by an obstruction of the pores.[17] But in this case we do not have to deal with an adsorbable substance. It still remains an open question for the future, how far the decrease of cell-permeability during narcosis, so often already observed, is to be attributed to the porosity of the plasma membrane. If this really happens, our ideas as to the nature of narcosis would be greatly supplemented.

Now we will consider an especially difficult matter. As we have seen, the entrance of numerous organic non-electrolytes into the living cell may be considered as a matter of lipoid-solubility; the entrance of other organic non-electrolytes, of some ions and of water may be considered as a matter of diffusion through the pores of the plasma membrane. But there is a group of substances of a very remarkable physiological significance, which can neither directly enter by dissolving in the oily phase nor by migrating through the porous phase, but which, nevertheless, do obviously enter. To this group belong substances which constitute a considerable proportion of the nutritive material, such as many sugars and amino-acids. There can be no doubt that this passage is not merely a simple form of permeation, in the sense that it depends on a certain permanent and invariable physicochemical behavior of a membrane. Either the plasma membrane must change under definite conditions in such a way that a temporary removal of the barrier to diffusion is brought about, or,—as has often been supposed,—reversible chemical reactions of the foodstuffs occur even in the surface of the cell, so that either the products of reaction are enabled to pass through or a more or less complicated series of single reactions is terminated by the appearance of the foodstuffs inside the cell wall.

Adhering to the physicochemical character of this lecture, we will discuss, basing our remarks on experimental data, only one of these forms of the ingestion of the nutritive substances, namely the alteration of the plasma membrane in such a way that for a short time it becomes permeable to substances for which it is otherwise not permeable.

As a matter of fact, there is a well-known form of intake of nutritive material which can be considered as an opening of the plasma membrane, that is, phagocytosis. For as the protoplasm is flowing around the particles of food in order to incorporate them, the superficial layer necessarily must be partly destroyed. On the other side there exist further conditions for naturally opening the plasma membrane in a reversible manner, particularly as a so-called functional increase of permeability, that is, as an increase of permeability accomplished by function, or better, by excitation as preparation for function. I am not able to give an extended review of our knowledge of functional increase of permeability within the limits of this lecture, but I shall relate one striking demonstration of the bringing about of a reversible increase of permeability. If one brings *Spirogyra* cells into a solution of cyanol, a well-diffusing blue sulfonic acid dye, the protoplasts will remain unstained for several weeks. Some years ago in my laboratory Banus observed that while sending an alternating current of appropriate strength through the threads of algæ, the blue dye would pass out of the solution into the interior of the cell, namely into the sap of the vacuoles.[18] After this, the current being stopped, the algæ were left for some time in the blue solution; then they were taken out and washed with pure water. It resulted that the vacuole retained the blue dye in spite of its diffusibility, the dye which entered being imprisoned as long as the cell was alive. Apparently the electric current had opened the plasma membrane, a substance to which the interior of the cell is closed under natural conditions had penetrated, and behind it the plasma membrane had shut up. In this way an event was produced, owing to experimental conditions, that is never realized in nature; but a natural phenomenon, the reversible increase of permeability, had been reproduced, possibly in a somewhat crude manner. Perhaps there occurred only a regeneration after an injury generated by the current. But, examining the conditions more closely, we may recognize that nature may sometimes duplicate them. For, in regard to the well-known studies of Bethe and Toropoff on gelatine diaphragms, it is highly probable that the flow of an electric current is accompanied by changes of hydrogen and hydroxyl ion concentration on the cell boundary so that these active ions, either by hydration and liquefaction or by aggregation of the surface colloids, can amplify or narrow the paths to be taken by diffusing substances and can in this way produce reversible changes in permeability.

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[17] Michaelis and Weech, *Jour. of Gen. Physiol.*, **12**, 55, 1928.

Thus we learn more and more to regard the plasma membrane as a formation with varying properties so that its permeability exhibits different degrees succeeding one another in time. But the plasma membrane does not only vary in one and the same object temporarily, but,—and this shall be the last point to be discussed in this lecture,—it varies also in one and the same kind of cell from species to species.

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[18] Banus, *Pflüger's Arch.*, **202,** 184, 1924.

I shall only demonstrate this with one especially simple object, namely, the red blood corpuscles again, and with this object I wish to demonstrate further in what direction the research into the nature of cell permeability is to be extended. Finally I return in this way once more to the phenomena of porous permeability and of solution permeability of the cells.

As we have seen before, the limiting membrane of the blood corpuscles, according to the electropositive charge in the wall of its pores, allows only the anions to exchange by diffusion from one side of the membrane to the other. Further, it has been pointed out by different authors that each anion passes through the corpuscle membrane with a specific velocity. Now Mond in my laboratory has raised the question of the existence of differences in the relative velocities from species to species as evolving from the different sizes of the holes in the sieve-like membrane, and in order to decide this question, he examined the exchange of chlorine ions against sulfate ions, which are known to wander especially slowly.[19] Mond actually found considerable differences in the different animals. The interchange is quickest in the blood corpuscles of man, then there follow pig, horse, cattle. The conclusion that we have to come to in the experiment just described with differences in pore size has been supported by Mond by comparing the sulfate ion with the tetrahydric alcohol erythritol as a non-electrolyte which is insoluble in lipoids and which is known to penetrate into the blood corpuscles and other cells as slowly as the sulfate ion. The same result occurred, namely, the speed of permeation was greatest with the corpuscles of man and the least with those of cattle.

But there exist not only differences from animal to animal in the porous permeability of the cells; the same state of affairs holds for the solution permeability. It is well-known that almost every basic dye enters the living cell, but there are rather few acid dye-stuffs that are suitable to it. As to the sulfonic acid dyes, evidently only those enter which dissolve in the oil mixture worked out by Nirenstein and, as has been illustrated by a table in the beginning of my lecture, the dyes enter the cells the more as their relative solubility in the oil mixture is greater. Now I have discovered that the partition coefficient of blood corpuscles to surrounding solution differs under the same conditions from one species to the other; for example, the coefficient is greater with the blood corpuscles of the pig than with those of cattle and sheep, and with these greater than with those of the horse.[20] This is demonstrated for two dyes in Table V.

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[19] Mond and Gertz, *Pflüger's Arch.*, **221**, 623, 1929.

[20] Unpublished experiments.

It appears at once that two explanations may be attempted: either we have to assume that the blood corpuscles of all four animals contain lipoids of the same quality, on which the dyes are distributed, but the quantity is greatest in the corpuscles of the pig and is smallest in the corpuscles of the horse; or we have to do with nearly the same quantity of the lipoids in every kind of corpuscle, but the lipoids differ qualitatively as to their power to dissolve dyes, the power being greatest with the pig and smallest with the horse. It is my opinion that we must prefer the second explanation; for whenever the passage of the dyes is dependent here upon the lipoid solubility,—and unquestionably this is the case,—then we must expect that a dyestuff penetrating into the blood corpuscles of the pig will also get through the corpuscles of the horse, even if their lipoid phase is very small; but I have found that, on the contrary, the corpuscles of the horse are nearly impermeable to several of the staining substances examined. Thus we conclude that not only the properties of the porous phase of the cell boundary, but also its dissolving properties, vary from animal to animal.

Table V

Partition of Dyestuffs to Blood Corpuscles

Kind of Corpuscle	Dye	Initial Concentration	Final Concentration	Partition Coefficient
Horse	Tropæolin 1	0.0025	0.0017	1.9
Cattle	Tropæolin 1	0.0025	0.0015	2.7
Pig	Tropæolin 1	0.0025	0.0013	3.7
Horse	Bromophenol blue	0.0025	0.0022	0.55
Cattle	Bromophenol blue	0.0025	0.0020	1.0
Pig	Bromophenol blue	0.0025	0.0015	2.7

Ladies and gentlemen, I have come to the end and I shall repeat now the previously quoted words of Professor Jacobs: "It may be emphasized that what is most needed in the field of cell permeability at the present day is facts." And in relation to that I wish to add to what I have already said to you a quotation from Professor Ralph Lillie's lecture concerning the scientific view of life. He said: "What is required is the imagination or construction of some model that will reproduce in intelligible form the essential features of the phenomenon under consideration. Intelligibility is the essential criterion of the scientific view; it aims at making phenomena intellectually comprehensible." We perceive better than anywhere else the striking advantage of using models in the development of our knowledge of cell permeability, beginning with Traube and Pfeffer and passing from

Overton to Collander and Michaelis. It is very peculiar that in this direction the physical chemists have realized almost nothing from these weary, but very fascinating and instructive studies of what is required as a model of the cell membrane. This is to their own disadvantage, I believe, because they here overlooked fundamental problems worthy of pursuit by the methods of exact science which are applicable to the membranes, the qualities of which have been discussed in this lecture. Under these circumstances the physiologist is constrained, and will be constrained still more in the future, to leave his proper work, as he must for a shorter or even for a longer time put away physiology, and become pure physicist or pure physical chemist in order to answer preliminary questions of great importance to physiology. Otherwise he will be open to the great danger of fabricating hypotheses. But whoever among the physiologists resolves to leave his physiological studies, he may encourage himself by remembering that it was Jacques Loeb who, feeling obliged to do so in regard to his science and to himself, created in the last years of his life a monumental work on the physical chemistry of the protein bodies. In this way he manifested anew his perseverance and his enthusiasm, both properties which distinguished also Reynold Spaeth, whose memory is with us today.

THE COPPER CONTENT AND THE MINIMAL MOLECULAR WEIGHT OF THE HEMOCYANINS OF *BUSYCON CANALICULATUM* AND OF *LOLIGO PEALEI*

HUGH MONTGOMERY

(*From the Department of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole*)

The view, originally put forward by Fredericq (1878), that copper is a normal constituent of hemocyanin and that it has a significance in the respiratory function of this protein similar to that of iron in hemoglobin has been substantiated by later investigations, particularly those of Begemann (1924) and Redfield, Coolidge and Montgomery (1928), which show that the combining ratio of copper to oxygen is the same in the blood of a large number of invertebrates. A knowledge of the quantity of copper in hemocyanin consequently provides significant information with regard to its respiratory function. Inasmuch as the amount of copper in the various hemocyanins does not appear to be the same, such data gives unequivocal evidence of the specific character of the respiratory pigments in the different groups of invertebrates. Furthermore, because of the very small number of copper atoms in the hemocyanin molecule, the copper content is a most valuable basis from which to estimate the minimal molecular weights of these proteins.

In this paper an investigation of the hemocyanin of the whelk, *Busycon canaliculatum*, and of the squid, *Loligo pealei*, is described. Mendel and Bradley (1906) studied the respiratory protein of the blood of the whelk, which they called hemosycotypin,—a name derived from the then current generic name of this form, *Sycotypus*. They report that it contained zinc as well as copper.[1] They concluded that copper composed only 0.043 per cent of the weight of the molecule, a value very much smaller than that obtained in the case of other hemocyanins and one which leads to very high estimates of the protein content of the blood when the oxygen capacities demonstrated by

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[1] It seems preferable to include "hemosycotypin" among the hemocyanins because it has been demonstrated that the combining ratios of copper and oxygen are the same in this case as in that of other hemocyanins and because recent observations in this laboratory appear to make it doubtful whether the zinc is a true constituent of the protein molecule. Inasmuch as specific differences appear to exist between the hemocyanins of different groups of animals, confusion will be apt to result if each hemocyanin is given a different specific name.

Redfield, Coolidge and Montgomery (1928) are taken into account. The copper content of the hemocyanin of the squid does not appear to have been previously examined.

The copper content of these hemocyanins has been determined on material purified according to several standard procedures applicable to protein substances. Analyses for copper were made by the method described by Redfield, Coolidge and Shotts (1928). Between 10 and 20 c.c. of the hemocyanin solutions were used in each sample. The samples were dried in an oven at 100–110° C. for 48 hours, cooled in a dessicator and weighed. This procedure was repeated daily until successive weights did not vary more than 1 mgm. The samples of dried hemocyanin weighed between 100 and 300 mgm. Digestion, the electrolytic separation of copper, and its estimation were carried out exactly as described, except that in the titration 15 drops of potassium iodide were used instead of 10, as this modification was found to sharpen the end point.

We have not succeeded in producing definitely crystalline preparations of the hemocyanin of *Busycon canaliculatum* by methods which have been found applicable in other cases. Dhéré, Baumeler and Schneider (1929) have also been unsuccessful in crystallizing this hemocyanin. However, on prolonged dialysis against distilled water a precipitate is formed which appears to be composed of short rods and which gives a silky sheen on shaking similar to that characteristic of crystalline protein preparations.[2] *Busycon* hemocyanin appears to be a globulin, as it is insoluble in the region of its isoelectric point in salt solutions of sufficient dilution. This property has been used in purifying our material as well as the usual procedure of salting out with ammonium sulphate, employed by Redfield, Coolidge and Shotts (1928) in the preparation of *Limulus* hemocyanin.

[2] In an attempt to produce crystals, a number of preparations of hemocyanin, all of which showed a silky sheen on shaking, have been made by different methods from several species. The precipitated particles were too small, however, to be recognized under the microscope as definite crystals, though a very fine rod shape was observed in many cases. By the addition of 2 drops of serum to 1–2.5 c.c. of 0.05M acetate buffer solution of pH 4 to pH 5, the hemocyanins of *Busycon canaliculatum* and of *Busycon carica* were precipitated and showed a sheen on shaking. In the case of the bloods of the eight different species; *Limulus polyphemus* (horseshoe crab), *Busycon canaliculatum*, *Busycon carica*, *Libinia emarginata* (spider crab), *Loligo pealei*, *Homarus americanus* (lobster), *Callinectes sapidus* (blue crab), and *Ovalipes ocellatus* (lady crab), the hemocyanin was precipitated by diluting the serum 20 to 200 times and adding a few drops of 0.006 per cent acetic acid to 5 c.c. of the diluted serum. The acid must be added slowly or a precipitate will be formed which will show no sheen. Too much acid redissolves the precipitate.

In several cases these hemocyanin precipitates were concentrated by centrifuging and redissolved, whereupon the solutions appeared distinctly blue. This color disappeared when the solution was reduced with sodium hydrosulfite so that evidently the hemocyanin was not denatured by the process.

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TABLE I

Copper Content of Hemocyanin of Busycon canaliculatum

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
IVa	Three washings at isoelectric point	0.2071	0.496	0.240
		0.2053	0.496	0.242
		0.2067	0.492	0.238
		0.2070	0.482	0.234
IVb	Four additional washings at isoelectric point	0.1541	0.366	0.237
		0.1538	0.378	0.245
		0.1544	0.371	0.240
		0.1554	0.378	0.243
VI	Three washings at isoelectric point	0.0914	0.225	0.246
		0.0919	0.204	(0.227)
		0.0916	0.217	0.238
		0.0917	0.230	0.238
		0.2694	0.642	0.238
		0.2699	0.634	0.235
		0.2698	0.633	0.235
		0.1816	0.437	0.241
		0.1821	0.433	0.239
VII	Salting out and dialysis	0.1694	0.440	0.260
		0.1680	0.436	0.260
		0.1700	0.441	0.260
		0.1699	0.443	0.260
		0.1693	0.436	0.258
		0.1693	0.438	0.258
		0.1693	0.434	0.256
		0.1693	0.440	0.260
		0.1693	0.441	0.260
		0.1693	0.434	0.256
VIII	Salting out and dialysis under conditions leading to precipitation	0.1075	0.263	0.242
		0.2130	0.517	0.242
		0.2120	0.530	0.250
X	Salting out and dialysis	0.3967	0.948	0.239
		0.3948	0.944	0.238
		0.3965	0.950	0.238
		0.3978	0.948	0.237
		0.6492	1.535	0.236
		0.6485	1.554	0.240
		0.6487	1.534	0.236
XI	Salting out and dialysis	0.5494	1.318	0.240
		0.5481	1.308	0.238
		0.5483	1.309	0.239
		0.5478	1.311	0.239
		0.5472	1.315	0.240

Specimen IVa was made from blood which had been preserved with toluene in the cold room for two weeks. It was diluted with ten times its volume with distilled water and brought into the region of its isoelectric point by the careful addition of 0.01N HCl. The precipitate resulting was separated by centrifuging and put into solution in the original volume of water by the addition of an amount of sodium hydroxide equivalent to the hydrochloric acid previously added. This process was twice repeated. The precipitate finally obtained was washed with distilled water. The final product contained only a trace of chloride. Whenever acid or alkali was added, it was run in through a glass tube which had been drawn to a fine point while the hemocyanin was being vigorously stirred. In order to determine whether further purification of this product could be obtained, the entire process of purification was repeated four more times on a portion of Specimen IVa, the resulting preparation being designated Specimen IVb. Specimen VI was made in a manner similar to Specimen IVa. Specimen VII was made from blood which had been preserved half-saturated with ammonium sulphate for a month. The precipitated hemocyanin was separated by centrifuging and dissolved in a large volume of 5 per cent saturated solution of ammonium sulphate. The solution was centrifuged in order that a small amount of insoluble material might be discarded, and the solution was reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was then dialyzed against 0.001N sodium hydroxide under 20 cm. Hg reduced pressure for two weeks, at the end of which time it was free of sulphate. The preparation of Specimen VIII included the same steps as Specimen VII, except that it was dialyzed against 0.001N sodium hydroxide for five weeks at atmospheric pressure. At the end of the fifth week a precipitate appeared in the solution which gave on shaking a silky sheen similar in appearance to that produced by protein crystals. The precipitate consisted of rod-shaped particles about 2 μ in length. The solution still contained traces of sulphate and was consequently centrifuged and the precipitate washed three times with a large volume of distilled water. The sulphate test was then negative. Specimens X and XI were prepared from material which had been kept over two years precipitated in half saturated ammonium sulphate. They were purified by reprecipitation with ammonium sulphate (pH 8.0), repeated three times, followed by dialysis against 0.0001 sodium hydroxide for 18 days. The preparation and analysis of Specimens X and XI were made by Miss Elizabeth Ingalls.

The results of the analyses of these preparations are given in

Table I. The copper content obtained in the case of preparations made in the various ways is very nearly the same. This fact may be taken as evidence that fairly pure preparations of the protein have been obtained. The fact that the copper content of Specimen IVb was not materially increased over that in Specimen IVa by additional washing is further evidence for the adequacy of the method of purification employed.

The best representative value of the copper content of *Busycon canaliculatum* hemocyanin appears to be 0.24 per cent. Specimen VII yields consistent values 0.02 per cent higher than this. Inasmuch as Specimens VIII, X and XI, prepared by the same general method, agree with the general series, it is probable that the high value obtained in the case of Specimen VII should be attributed to some systematic analytical error rather than to superiority in the method of preparation.

Two specimens, which were obtained by the dialysis of fresh blood without other attempt at purification, yielded a product which contained about 0.22 per cent copper. This material was free of chloride and had the same nitrogen content per unit weight as the others. The result would appear to indicate that another protein may be present in the blood, but that if so, it exists only in small amounts. In the case of *Limulus*, the hemocyanin appears to account for about 95 per cent of the protein of the serum. In order to investigate this possibility further an attempt has been made to determine how far the nitrogen content of the blood of *Busycon canaliculatum* may be accounted for by the hemocyanin contained in it as estimated from the quantity of copper present. The nitrogen content of Specimen X was determined by the Kjeldahl method. Successive analyses yielded 15.6; 15.5; 15.7; 15.5; 15.4; 15.7; mean 15.5 grams nitrogen per 100 grams dry weight. The copper content of Specimen X was 0.238 grams per 100 grams dry weight. One part of copper consequently corresponds to 65.2 parts of nitrogen. Two specimens of blood were analyzed for copper and nitrogen. The first contained 0.074 mgm. copper per c.c. and 4.92 mgm. nitrogen per c.c. From the copper content it may be estimated that it contained 4.84 mgm. nitrogen as hemocyanin. The second specimen of blood contained 0.066 mgm. copper per c.c. and 4.14 mgm. nitrogen per c.c. The hemocyanin concentration as estimated from the copper content would account for 4.3 mgm. nitrogen. It is evident from these measurements that hemocyanin will account approximately for all of the protein nitrogen in *Busycon* blood.

One preparation of the hemocyanin of the allied species, *Busycon* *carica*, was made. The blood had been preserved in a precipitated condition in half-saturated ammonium sulphate for one year in the cold room. The hemocyanin was separated, purified by the procedure employed in the case of *Busycon canaliculatum* Specimen X. Analysis of the copper content of the purified material yielded the following values: 0.217, 0.235, 0.238 per cent. The copper content of the hemocyanin of this species appears to be approximately the same as that of *Busycon canaliculatum*.

The hemocyanin of the squid, *Loligo pealei*, may be readily crystallized by methods similar to those first employed by Henze (1901) in preparing crystalline *Octopus* hemocyanin, and consequently lends itself well to purification. Squid hemocyanin is insoluble in solutions containing high concentrations of ammonium sulphate. It was found that if enough saturated ammonium sulphate solution is added to the blood to form a very slight cloud of precipitated hemocyanin, a fuller precipitation in the form of crystals can then be produced by several procedures designed to decrease the solubility of the hemocyanin in the solution. These were: (1) the careful addition of increasing quantities of ammonium sulphate, (2) increasing the hydrogen ion concentration as in the Hopkins-Pinkus (1898) method of crystallizing albumen, or (3) raising the temperature. These methods can be used with success in combination. Crystallization by raising the temperature, which is presumably due to increasing the "salting out" effect of the ammonium sulphate at the higher temperature is particularly efficacious and has the advantage that it involves the addition of no reagents and may consequently be accomplished slowly so as to favor the formation of crystals. It was found that by raising the temperature from 0° C. to 30° C., a heavier crystalline precipitate is produced than by raising it to room temperature only. A temperature change within a range which will not denature the protein did not crystallize all the hemocyanin that was in the solution. Consequently, the yield may be increased by combining the temperature method with the addition of ammonium sulphate or of acid. When crystallization is produced in this manner, there is formed first a fine precipitate, visible under the microscope but apparently amorphous. This changes in a few minutes to fine rods and then to bundles of needles and finally to large needles. The process is much like that described in the case of *Eledone moschata* hemocyanin by Kobert (1903). The appearance of the crystalline rods is similar to that figured by Dhéré (1919, figure 4), in the case of the oxyhemocyanin of *Helix pomatia* formed in the presence of sodium sulphate. If large excess of reagents are added suddenly, the precipitate produced is amorphous. Crystallization of squid hemocyanin was obtained more readily from fresh blood than from preparations which had been preserved in a precipitated condition in concentrated ammonium sulphate or from previously crystallized hemocyanin. Crystals which had been kept for a year in the cold room in their mother liquor (half saturated ammonium sulphate), were found to have become insoluble in distilled water. This phenomenon was observed by Craifaleanu (1919) in the case of crystals of the hemocyanin of *Octopus vulgaris*. Craifaleanu called this form "para-hemocyanin."

Table II

Copper Content of Hemocyanin of Loligo pealei

Specimen No.	Method of Preparation	Dry Weight	Copper	Copper
		grams	*mgm.*	*per cent*
I	Salting out and dialysis	0.1485	0.384	0.258
		0.1486	0.371	0.250
		0.1502	0.388	0.257
		0.1490	0.376	0.252
II	Crystallization and dialysis	0.0785	0.194	0.244
		0.1620	0.386	0.238
		0.1624	0.390	0.242
V	Salting out and dialysis	0.4579	1.155	0.252
		0.4594	1.161	0.254
		0.4593	1.178	0.256
		0.4601	1.159	0.252
		0.4592	1.154	0.252

Analyses of the copper content of the hemocyanin of *Loligo pealei* have been made upon three preparations. Specimens I and V were prepared from blood which had been precipitated by the addition of ammonium sulphate to half saturation and kept in the cold room at about 5° C. for two years. The material had a fishy odor, which disappeared when it was shaken with air and from which the final preparations were entirely free. The precipitate was separated from the supernatant fluid with the centrifuge and was dissolved with a small volume of 5 per cent ammonium sulphate. The solution was again centrifuged to throw down any insoluble material, and the fluid was drawn off and reprecipitated by the addition of saturated ammonium sulphate. This process was repeated twice. The solution was finally dialyzed until it was found to be free of sulphate. Specimen II was prepared by crystallization from fresh blood. The blood was chilled to 0°, and then sufficient saturated ammonium sulphate was added to produce a very slight precipitation of hemocyanin. The temperature was then raised from 0° to 20°, when full precipitation was obtained. The precipitate was in the form of needle-shaped crystals about ten μ in length. The crystals were separated from the mother liquor by centrifuging and dissolved with 5 per cent saturated ammonium sulphate. Insoluble material was removed by centrifuging, and the hemocyanin was then reprecipitated as before. This second precipitate was not crystalline, however. The preparation was then dialyzed against water until free of ammonium sulphate. All three preparations had a clear blue-green color and became colorless in the characteristic way upon reduction with sodium hydro-sulphite.

Table II contains the data obtained from analyses of these preparations of squid hemocyanin, which all yield values for the quantity of copper in the molecule close to 0.25 per cent.

It is interesting to compare the values obtained for the copper content of the hemocyanin of *Busycon* and *Loligo* with those previously reported for other species, particularly with regard to their systematic relationships. In Table III are collected the various determinations of the copper content of hemocyanin which occur in the literature. It is noteworthy that the value obtained in the case of *Busycon canaliculatum* and *Busycon carica* does not differ greatly from those attributed to the other gastropod, *Helix pomatia*. The value obtained for *Helix pomatia* by Begemann, whose method of copper analysis we have employed, exceeds the value obtained with *Busycon* by an amount well in excess of the apparent experimental errors. These hemocyanins appear also to differ in certain other respects. *Busycon* hemocyanin cannot be crystallized by methods which succeed in the case of *Helix* (Dhéré, Baumeler and Schneider, 1929). *Busycon* hemocyanin is insoluble in the region of its isoelectric point in the presence of quite

TABLE III

	Copper	Author
	per cent	
Cancer..................	0.32	Griffiths (1892).
Homarus...............	0.34	"
Sepia..................	0.34	"
Octopus vulgaris.........	0.38	Henze (1901).
Loligo pealei............	0.25	
Helix pomatia..........	0.25	Burdel (1922).
" "	0.29	Begemann (1924).
Busycon canaliculatum...	0.24	
Limulus polyphemus.....	0.173	Redfield, Coolidge and Shotts (1928).

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appreciable amounts of salt. *Helix* hemocyanin, on the other hand, appears to be readily dissolved by very small concentrations of salt under these circumstances (Svedberg and Heyroth, 1929).

It is surprising that such a great difference exists between the copper content of the hemocyanin of the squid and that of the octopus. Inasmuch as the properties of the respiratory pigments in these two cephalopods appear to be very similar, we believe it to be desirable to redetermine these values by methods of preparation and analysis which are strictly comparable.

The weight of hemocyanin containing one atom of copper is given by dividing the atomic weight of copper, 63.57, by the fraction of the weight of hemocyanin due to this element. In the case of *Busycon canaliculatum* this fraction is 0.25×10^{-2}. The minimal molecular weight of *Busycon* hemocyanin thus appears to be approximately 26,500, when estimated upon the basis of its copper content. It has been shown, however, by Redfield, Coolidge and Montgomery (1928), that when hemocyanin becomes associated with oxygen to form oxyhemocyanin, one molecule of oxygen is combined with a quantity of hemocyanin containing two atoms of copper. Inasmuch as it appears highly unlikely that the oxygen molecule is dissociated into its constituent atoms in its reaction with the respiratory protein, it seems safe to assume that each molecule of oxyhemocyanin is combined with not less than one molecule of oxygen. The hemocyanin molecule must consequently contain at least two atoms of copper. Estimated on this basis, the minimal molecular weight of *Busycon* hemocyanin is approximately 53,000. In a similar way it may be calculated that the minimal molecular weight of the hemocyanin of *Loligo pealei*, estimated on the basis of its copper content, is 25,400, and when the oxygen-combining relations are taken into account, the combining weight appears to be approximately 51,000.

SUMMARY

The hemocyanin of *Busycon canaliculatum* contains 0.24 per cent of copper and 15.8 per cent of nitrogen. Its minimal molecular weight is approximately 53,000.

The copper content of the hemocyanin of *Busycon carica* appears to be the same.

The hemocyanin of *Loligo pealei* contains 0.25 per cent of copper and has a minimal molecular weight of approximately 51,000.

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GROWTH AND DIFFERENTIATION OF THE COLONIES OF *ZOOTHAMNIUM ALTERNANS* (CLAP. AND LACHM.)

E. FAURÉ-FREMIET

Collège de France, Paris

Introduction

In a preceding publication (1922) I have insisted on the fact that colonial Vorticellidæ constitute an intermediary step between a population of like cells (cultures of free Infusoria) and a multicellular organism; unlike free cells with unlimited power of division, whose population growth theoretically follows a geometrical progression. The colonies of *Epistylis,* of *Carchesium,* or of *Zoothamnium* generally have a limited growth, following a special cycle, independent of a possible sexual cycle. In these colonies the lineage of each cell is perfectly defined by dichotomous ramifications of a common peduncle, and it is possible to show in a large number of cases the existence of somewhat differential divisions giving two sister cells whose power of multiplication is different. In certain species (*Epistylis arenicolæ, Epistylis Perrieri*) the first divisions can be dichotomous and equal, so that the mass growth of a number of individuals follows a geometrical progression; but soon the sister cells resulting from each division multiply unequally, and the growth approaches more or less an arithmetical progression.

On the other hand, the study of the growth of the common peduncle, which is considered as a product of the protoplasmic activity, shows that the latter may decrease in course of time. But the Vorticellidæ colonies form, from time to time, migrating individuals which may be of the same size as the other individuals (*Carchesium, Epistylis*) or more voluminous (some *Epistylis,* some *Zoothamnium,* called heteromorphic). In these individuals, and in these only, appear secretory granules already observed by Engelmann and more recently (1926) by Wesenberg-Lund, which seem to be connected with the formation of the peduncle, and one can consider the hypothesis of an active substance, or of a transformable substance, produced in a definite quantity and periodically, by certain individuals, which is divided among the descendants of the latter and at the same time is diminished little by little.

It appears then that the growth of a group of cells may be limited by factors somewhat internal but altogether independent of the hypothetical notion of a "factor of senescence"[1]

The *Zoothamnium* called heteromorphic, about which I have given some detail in my paper of 1922, seems to give the most typical examples as to the rôle of these internal, or properly cellular factors, in the general form of growth of a colony and its limitation.

Claparède and Lachmann described in 1858 a marine species, *Zoothamnium alternans* (described later by Möbius under the name of *Z. Cienkowskii*); the aspect of the colonies, they say, is that of "un arbre à branches courtes et très régulièrement alternantes. La forme de ces familles a sa cause dans un arrêt de division spontanée qui frappe en général l'un des deux individus issus de chaque division. Lorsqu'un individu *A* se divise en deux individus *B* et *B′*, l'un des deux, *B* par exemple, ne se forme qu'un pédoncule fort court et son développement reste stationnaire à partir de ce moment, tandis que l'autre, *B′*, secrète un pédoncule plus long, puis se divise en deux nouveaux individus, *C* et *C′*, dont le premier, qui est toujours du côté de la branche opposée à celui où se trouvait l'individu *B*, ne forme qu'un pédoncule très court et ne se divise pas davantage tandis que *C′* forme un pédoncule plus long et se divise en deux individus *D* et *D′* et ainsi de suite."

That is not all; in *Z. alternans* and in *Z. arbuscula* Ehrb. or *Z. geniculatum* Ayrton (see Wesenberg-Lund, 1925, and Furssenko, 1925) the migrating individuals which will be the origin of new colonies and will thus begin a new cycle, are distinguished not only by a few morphological characters, but also by their voluminous size and the well-determined place where they originate in the colony, generally at the junction of the main branches. These large migrating individuals are the "ciliospores" of Wesenberg-Lund or "macrozoides" of Furssenko, much larger than the "trophozoides" or "microzoides" which constitute the most numerous individuals of the colony.

Ehrenberg had observed these individuals in *Z. arbuscula,* and had noticed that they result from the growth of an individual not unlike the others, but always situated at the junction of a branch. This author admits that one of the two individuals issued from a bipartition on the branch while the other grows without dividing, thus being, he says "the aunt" of the individuals of the branch. Claparède and Lachmann find this same condition in *Z. alternans,* but sometimes this growing individual may undergo a division. *Zoothamnium alternans* (Claparède and Lachmann) is found frequently on the coasts of Brittany; I have found it in abundance in Woods Hole and was able to follow the different stages of the colony cycle and of the formation of the "ciliospores." I observed a few phenomena of conjugation, quite sporadic, but I have not observed a sexual cycle analogous to the one discovered by Wesenberg-Lund in *Z. geniculatum* or described by Furssenko in *Z. arbuscula.*

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[1] In other publications (1925–26) I tried to show that in several very different cases the idea of a factor of senescence could be replaced either by the hypothesis of differing speeds in a group of transformations necessary to cellular activity, or by the assumption of a "probability" of transformation which would be too long to develop here. (See Fauré-Fremiet and Laura Kaufman, 1928, and Fauré-Fremiet and H. Garrault, 1928.)

Technic

In order to follow the complete evolution in a large number of colonies, I have used numbered slides, ruled in squares with a diamond point. These slides were first placed in a crystallization dish containing numerous colonies of *Z. alternans.* After several hours, they were removed and placed in a Petri dish containing sea water and examined under a binocular microscope. All individuals recently attached were carefully located and designated in numeral order; those whose peduncle had already developed or had already given the first division were removed with a needle.

After this operation, the slides were placed vertically on frames floating in an aquarium through which ran a strong current of sea water; this was done to avoid the deposit of particles and of microörganisms. The slides were then examined periodically and the different stages of the development of each colony were carefully recorded in function of time.

When the cytological examination of a colony is necessary, it is always easy to detach this colony with a fine pipette, in order to study it under the high power, *in vivo,* or after fixation.

The best technic for the study of the nuclear apparatus is the fixation by OsO_4 for a short time followed by boracic carmine stain. The presence (generally in the Vorticellidæ) of a cuticle and the contractability of a peduncle constitute two technical difficulties which are not easy to overcome; it may be necessary to cut the colony with a fine scalpel in order to isolate certain individuals which it is necessary to fix and stain.

Structure of the Colonies

The appearance of colonies of *Z. alternans* is very nearly that of a palm (Fig. 1); they have a main trunk and oblique branches placed alternately in the same plane, on right and left of the axis; the main trunk always bears at the top a terminal individual of rather large size; the lateral oblique branches bear a variable number of small individuals;

finally along the trunk, at the juncture of the lateral branches, are found the voluminous migrating individuals either macrozooids or macrospores.

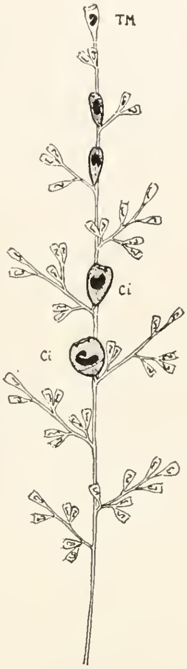

FIG. 1. A young colony of *Zoothamnium alternans* (Clap. and Lachm.), showing the main trunk and the alternate lateral branches. *TM,* terminal macrozooid; *Ci,* ciliospores at different stages of growth, located on the anterior side of the colony at the first division of each branch *D, E, G, H*. The branch *F*, in this case, bears, at the same place, only two microzooids apparently identical with the others.

The lateral branches of the colony observed in extension are almost always slightly curved in, and most of the individuals borne by these branches are inclined toward the outside of the curvature. The two sides of the palm are thus different, and one can define at the same time a base and a summit, an anterior and a posterior side.

The elements of symmetry of such a colony are a main axis represented by the trunk, and a median plane, antero-posterior, separating the two halves right and left.

As for all the other species of the genus *Zoothamnium,* the colonial peduncle bears an elastic tube whose rôle is passive, and a continuous " cordon central," dichotomically ramified, which represents the prolongation of the lower extremity of each individual; this central cordon has itself a protoplasmic tube (*I*) limited by a fine film and surrounding a muscular fiber which terminates at the basal part of each individual by a conical group of myonemes.[2] The migrating individuals, or "ciliospores," when liberated swim rapidly with their posterior ciliary crown. They are large individuals, flattened in the antero-posterior direction, and look like a top. They attach themselves by means of the scopula (*I*) and begin to secrete the peduncle. At the same time they lose their posterior ciliary crown and progressively take on again the ordinary subconical form.

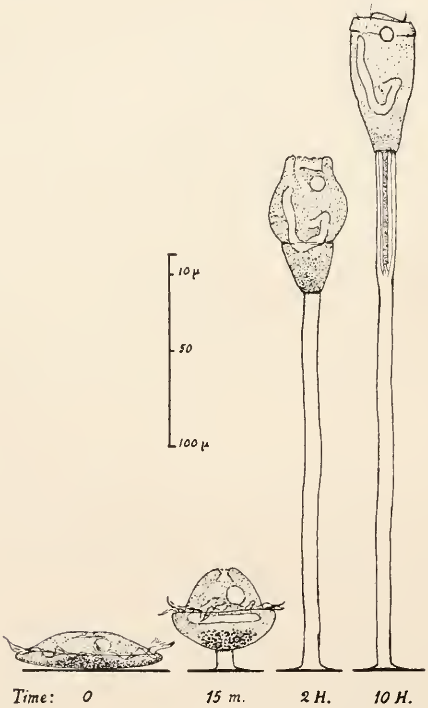

FIG. 2. Fixation of the ciliospore and construction of the peduncle. At first the top-like ciliospore turns quickly on the slide, then the building of the peduncle begins; the same individual is shown fifteen minutes after fixation. The ciliary crown slows down and disappears while the peduncle grows (Epistylis stage) during a short time (two hours); finally, one can see the differentiation of the "cordon central" and the muscular fiber (ten hours).

The peduncle is at first a solid cylindrical body of a fibrillar structure which grows rapidly ("Epistylis stage"); after two hours it reaches a length of about 250 μ. The secretion then begins to slow down and a section of the peduncle is ring-like; there is a central canal, at the bottom of which remains attached a part of the body of the infusorian, which from now on will lengthen itself along with the tube of the peduncle and become differentiated in a central cordon with the muscular fiber or "spasmoneme" (Fig. 2).

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[2] For the structure of the Vorticellidæ in general, and of the peduncle in particular, see Fauré-Fremiet (1906).

Six or seven hours (at the temperature of 21° C.) after the start of the secretion of the peduncle, the original individual undergoes a first unequal division which gives a macrozooid and a microzooid; the plane passing through these two zooids and the common peduncle is the median plane of symmetry of the future colony. The large cell remains clearly axial after this first division and continues to form actively the principal peduncle of the colony. After four to seven hours it undergoes a second unequal division; the interval between the following divisions is longer, from ten to sixteen hours; but always during the growth of the colony the terminal individual is a macrozooid, each division of which separates a microzooid in the median plane of the colony. The successive series of terminal microzooids constitutes a main strain perfectly schematized by the axial trunk of the colony.

We shall designate each cell of this series by a Roman numeral representing the division which started it; we shall have then the original individual, or ciliospore, then the series of macrozooids, I, II, III, . . . X, etc.

We shall designate with capital letters the corresponding series of median microzooids detached from the main strain (microzooids of first order), *A, B, C, . . . J,* etc. Each branch of the colony is started by the division, alternately at the right and at the left of the median plane of each microzooid of the first order. But, in accordance with the diagram of Claparède and Lachmann, only one of the two cells resulting from such a division is the origin of a lateral limb; we shall designate it by a small letter preceded by the coefficient 1; the other cell remains median and will be designated by its capital letter preceded by the same coefficient 1.

At the beginning of the formation of the fifth branch, for example, we shall have first the division of the terminal macrozooid IV, which will give a new terminal macrozooid V and a median microzooid *E*. The latter will divide in a perpendicular plane to that of the division of IV, and will give two individuals, one of which, 1*E*, remains in the median plane while the other, 1*e*, situated for example at the right of this plane, will be the origin of the branch (Fig. 3).

Each branch has also a main axis and lateral branches but does not have a well-defined median plane nor median individuals. The division of $1e$, for instance, gives rise to two cells apparently similar, $2e^1$ and $2e^2$. The individual $2e^1$ remains in the axis and gives at the new division $3e^1$ (axial) and $3e^2$ (lateral); $3e^1$ will give $4e^1$ (axial) and $4e^2$ (lateral), etc.

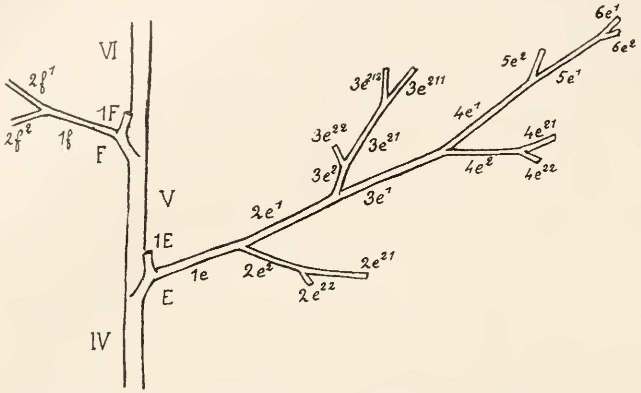


FIG. 3. Scheme of the branch *E* and the basis of the branch *F*, showing the lineage of the median microzooids 1*E* and 1*F* and the different microzooids.

Likewise the individuals $2e^2$, $3e^2$, and $4e^2$ will give successively two or three generations, the elements of which we shall designate by the symbols $2e^{21}$, $2e^{22}$, $3e^{21}$, $3e^{22}$, etc; according to the rule of Claparède and Lachmann $3e^{22}$ does not divide, but $3e^{21}$ gives $3e^{211}$ and $3e^{212}$; the number of generations formed by the lateral branches seems to be always rather limited.

The median individuals of the second generation: 1*A*, 1*B*, 1*C* . . . 1*E*, etc., can divide once and give $1A^1$ and $1A^2$ for example. But while 1*A*, 1*B*, 1*C*, and their two immediate descendants remain microzooids identical to these designated by the small letters, 1*D*, 1*E* and the following ones, or the two cells of the second generation, $1D^1$, $1D^2$; $1E^1$, $1E^2$, etc., undergo a considerable growth and are transformed into ciliospores, or migrating macrozooids, which soon detach themselves from the common trunk to swim freely and to attach themselves later on.

It appears clearly then that during the growth of a colony of *Zoothamnium alternans* the two cells resulting from the division of one initial cell are never equivalent as to their "potentialities." But in confirming the observations of Ehrenberg and of Claparède and Lachmann, we may now make them more precise by showing that the progressive segregation of the power of multiplication and of the power of growth is very rigorously tied up with the respective position of the individual separated by the successive divisions. It seems then that a certain number of divisions at least must be considered as differential divisions. The cytological examination confirms this interpretation.

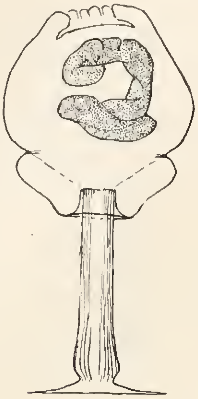
First Division of the Initial Macrozooid

The first division is characterized, in a rigorously constant manner, by the unequal division of the macronucleus and of the protoplasm of the initial individual of the colony (Fig. 4) between the first two cells, the macrozooid *I* and the microzooid *A* (Fig. 5). A short time before this division, the macronucleus, which takes the shape of a long twisted rod, enlarges at one of its extremities in a compact mass. The other extremity is thin and often flattens slightly, and becomes elongated in the median plane of the individual. The two edges of this flat portion are often slightly thickened, so that a side view gives the impression of a structure in a horseshoe shape. The micronucleus remains near the thick extremity and soon lengthens into a spindle. Meanwhile the peristome and the scopula divide as well as the central cordon of the peduncle and soon an upper and a lower furrow, growing in depth toward each other, begin to separate two cells of very unequal size. The micronucleus completes its own division, then the macronucleus is divided unequally at the time when the two furrows join; the macrozooid (which remains the terminal individual on the axis of the colony) retains the thickened part of the macronucleus and a micronucleus; the microzooid (which becomes the first median individual *A*) retains the thin part of the macronucleus and a micronucleus (Fig. 6).

Considering the irregular shapes of the body and of the macronucleus in *Z. alternans*, it is impossible to calculate the corresponding volume and to establish the values of the nucleoplasmic relation. Nevertheless, it is clearly evident that the ratio N/P is greater in the microzooids than in the macrozooids, *i.e.*, the macronucleus is divided into two daughter cells even more unequally than the cytoplasm.

It is difficult to establish whether there exists a difference in composition between the two unequal extremities of the macronucleus divided between *I* and *A*. The " nuclear reaction " of Feulgen does not show any difference between these two parts, and their structure differs very little. Most frequently one can observe a linear orientation, in a continuous and parallel line of the chromatin granules (microsomes) in the thin part of the macronucleus which will be distributed by the division. On the other hand, the voluminous mass which remains in the macrozooid *I* shows an irregular distribution of its microsomes. This mass behaves as a chromatin reserve which would not be affected at all by the phenomena of division.

Supposing that the terminal condensation of the macronucleus represents a kind of segregation of the chromatin material, we shall describe this first unequal division as a differential quantitative and qualitative division.



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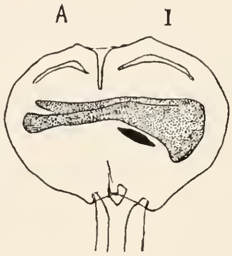

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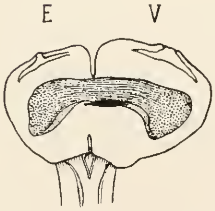

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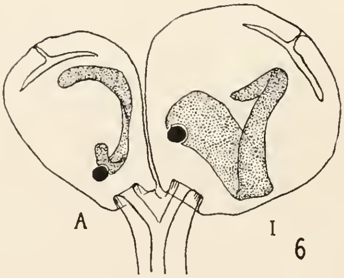

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Fig. 4. Ciliospores at the beginning of the peduncle's formation, showing the appearance of the macronucleus before the first division.

Fig. 5. First cleavage of the ciliospore, giving the terminal macrozooid *I* and the median microzooid *A*. The figure shows the differential division of the macronucleus (figured by dotting) and the apparently equal division of the micronucleus (black—spindle stage).

Fig. 6. Later stage of the first cleavage, showing the terminal macrozooid *I* and the median microzooid *A;* macronucleus figured by dotting; resting micronucleus black.

Fig. 7. Fourth cleavage on the main strain giving the terminal macrozooid *V* and the median microzooid *E*. The qualitative equal division of the macronucleus (figured by dotting) is shown.

Later Divisions of the Individuals of the Main Strain

The division of the individuals *I* and *II* presents exactly the same differential character as that of the initial individual. It is different at the time of division of the individual *III*. In the latter, the macronucleus shows at the outset of the bipartition a symmetrical thickening at each of its granular extremities which appear entirely homologous.

The median part, finely striated, is divided, however, into two unequal parts by the division of the protoplasmic body, which isolates here again an axial and terminal macrozooid, V, and a median microzooid E (Fig. 7).

All the later divisions of the individuals from the main strain, *i.e.*, *IV, V, VI . . . X* etc., are of the same type, and we shall consider these divisions as quantitatively differential only.

DIVISION OF THE MEDIAN MICROZOOIDS

The median microzooids, *A, B,* and *C,* which have received only the thin extremity of the initial macronucleus, undergo an almost equal division which gives for example $1A^1$ (median) and $1a^2$ (lateral) of the same dimension and of the same structure, both having a thin and twisted macronucleus, as well as the descendants of $1a^2$, $1b^2$, and $1c^2$ (Fig. 8).

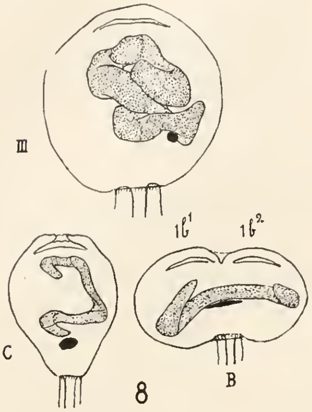
On the other hand, the median microzooids, *D, E, F,* and the following undergo an unequal division, quantitatively and qualitatively differential, like that of the first three individuals: the ciliospores *I* and *II.* A short time before the division, when the median individual begins to lengthen in the transverse plane, its macronucleus takes the shape of an elliptic blade, presenting in a marginal point a large subspherical thickening. This thick part of the macronucleus, on the other hand, lengthens at the time of division and is divided between the two individuals 1*D,* 1*d,* 1*E* and 1*e,* etc. (Fig. 9).

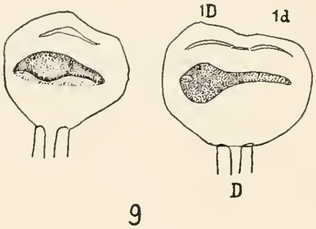
These facts indicate that the differential division takes place at two different times from the fourth generation of the axial cells. For instance, when the division of *III* divides into *IV* and *D,* the microzooid *D* has a little less than a half macronucleus; but this half macronucleus is qualitatively similar to that of the macrozooid *IV,* having a granular terminal thickening. However, the microzooid *D* shows a nucleoplasmic relation, a ratio N/P superior to that of macrozooid *IV,* for the protoplasm has divided much more unequally than the macronucleus. It is a small individual with a large macronucleus.

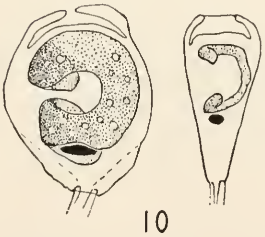
When the microzooid *D* divides, the cytoplasmic division is almost equal, but the division of the macronucleus is qualitatively differential, because the thickened and granular part does not divide but goes whole to the median individual 1*D.* The outcome is that the ratio N/P is still increased in this individual.

The axial microzooids 1*D,* 1*E,* etc., can undergo a division and give for instance $1D^1$ and $1D^2$; but these two individuals, which remain median, soon begin to enlarge without dividing any further.

The microzooids 1*d*, 1*e*, etc., as said above, go through a series of divisions which always give individuals with long and slender macronuclei.







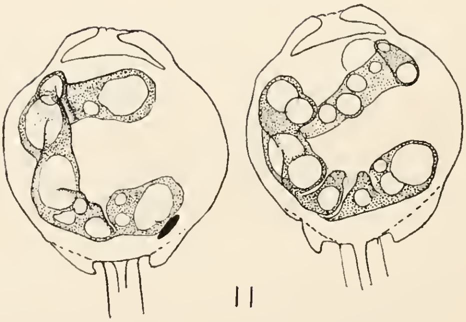


Fig. 8. Cleavage of the median microzooid *B*, giving, with equal division of the macronucleus, the microzooids $1b^1$ and $1b^2$. Comparison between the terminal macrozooid *III* and the median microzooid *C* (resting stage).

Fig. 9. Cleavage of the median microzooid *D*, giving the future median macrozooid 1*D* (ciliospore) and the microzooid 1*d*, with a differential division of the macronucleus.

Fig. 10. One median macrozooid (1*G* for example) at the beginning of its growth, and one microzooid of the corresponding branch. The large difference in size of the macronucleus is to be noted.

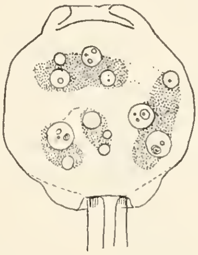
Fig. 11. Two median macrozooids during the time of growth. In the macronucleus, numerous large nucleoli are to be seen (figured as vesicles on the drawing).

Growth of the Median Microzooids and Formation of the Ciliospores

The median microzooids of the fourth generation (*D* or $1D^1$ and $1D^2$) and of the following generations (*E, F, G,* etc.) increase rapidly until they reach a length of about 55 μ to 70 μ, in one day, two days, or two and a half days.

The macronucleus, already voluminous, begins to grow and forms a very large horseshoe-shaped body. The micronucleus situated at the lower part in a slight depression lengthens into a spindle as in preparation for the division. While the macronucleus increases, rather refringent nucleoli appear in the midst of the chromatic granulations, not giving the reaction of Feulgen (Fig. 10).

Soon, while the protoplasmic growth goes on, it seems that the nuclear growth stops. The very numerous nucleoli alone still increase in volume (Fig. 11). Then the outline of the macronucleus disappears, the nucleoli project on the surface of the chromatic mass, and one can observe very numerous stages of disintegration and of degeneration of the macronucleus and of its fragments (Fig. 12).



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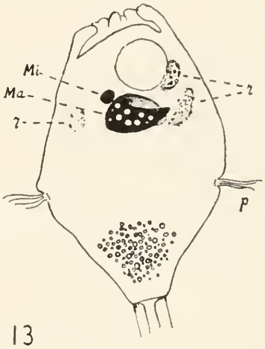

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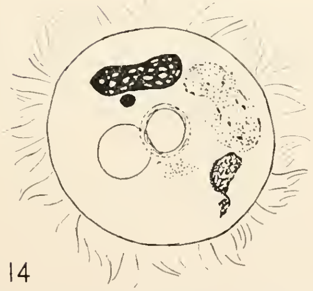

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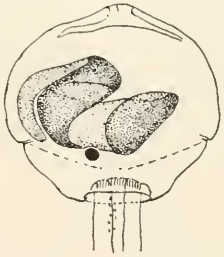

15

Fig. 12. Later stage of the median macrozooid's growth. Disintegration and disappearance of the macronucleus.

Fig. 13. One median macrozooid almost ready to leave the colony: *p*, posterior ciliary crown; *Ma* and *Mi*, macronucleus and micronucleus of the new nuclear apparatus; *r*, residual mass of chromatin.

Fig. 14. Top view of a median macrozooid (same stage as that shown in Fig. 12).

Fig. 15. Terminal macrozooid making the posterior ciliary crown and soon ready to leave the colony.

Finally, one sees in the center of the cytoplasmic mass containing a rather larger number of residual masses, a short macronucleus, arched, staining very intensely, containing only very small nucleoli, and accompanied by a resting spherical micronucleus (Figs. 13 and 14). This aspect, frequently observed, is that of a nuclear apparatus of new formation, and it is probable that the changes just described represent a phenomenon of endomixis. I was, however, unable to follow in the individuals stained *in toto* the fate of the spindle-shaped micronucleus observed in the preceding stages. It probably divides and makes up the new nuclear apparatus; but this stage was not observed in my set of preparations. At the end of the protoplasmic growth and when the nuclear changes are completed, a furrow appears around the median individual, at about the posterior third. It is the future ciliary crown, whose vibratile elements appear soon afterward. At the same time the organism flattens in the antero-posterior direction, and takes the shape of a top. The cytoplasm is filled with diverse inclusions, a great number of which are probably nuclear residue. In the posterior region, above the " scopula," appear very numerous inclusions which are not very refringent. Neutral red *in vivo* colors them a brownish red. These inclusions correspond to the secretion granules whose existence I have already mentioned in the migrating individuals of different Vorticellidæ.

There are still a few lipoid granules, and, toward the middle of the body, numerous small inclusions fixing neutral red in an intense red color. Iodine fixation gives a mahogany color, but the latter is not any stronger than for the microzooids.

The " ciliospore " which has thus been formed becomes almost lens-shaped. The peristome remains closed and the posterior ciliary fringe is animated with active movements which soon determine the liberation of the migrating individual (Fig. 15).

Growth of the Colonies of Zoothamnium alternans

At a temperature of 21° C., in an aquarium with running water, the growth of the colonies of *Z. alternans* goes on very regularly for a period of eight to ten days. Hence it is easy, by periodic examinations of a specific colony, to follow the increase in number of the individuals as a function of time. We have then a measure of the colony's growth. This measure is not very exact, because certain individuals grow without dividing and their mass is clearly larger than that of the others. However, the group of large cells given by the terminal macrozooid and the ciliospores is always rather restricted, and one can admit that the appearance of the development is rather well represented by the variation in number of the individuals. A more important error may arise from the fact that some parasitic Infusoria (*Acineta*) very often get into the microzooids (especially the microzooid of the first branch) and multiply in this individual, which does not divide and soon falls off.

Because of this, it is necessary at every investigation to trace a total scheme of the colony studied, indicating the place of each individual, which with some practice, may be quickly made by examining the colonies in extension in a thin water layer with a low power objective. By this means it is possible to keep an account of the accidental influences; but when the number of individuals increases too much, beyond the eighth day, for example, this method of pointing becomes very difficult and soon impossible to use with precision.

TABLE I

Date	Time	Numbers of Colonies Examined											
		1	2	3	4	5	6	7	8	9	10	11	12
July 14	11 A.M.	0	0	0	0	0	0	0	0	0	0	0	0
" 15	11 A.M.	III	III	III	III	II	III	III	III	II	III	III	
" 16	12 M.	V	V	V	IV	IV	III	IV	IV	III	IV	IV	
" 17	4:30 P.M.	VII	VIII	VII	V	VI	V	VII	VI	V	VI		
" 18	9 A.M.	VIII	IX	IX	VII	VII	VII	IX	VIII		VI		
" 19	10 A.M.	IX	XII		VIII	VIII	VIII	XI	X				
" 20	11:30 A.M.	XI	XIV		XII	X	IX	XIII					
" 21	9 P.M.	XII	XVI		XIV			XV					
" 22	9 P.M.		XVII			XII							

Date	Time	Numbers of Colonies Examined											
		13	14	15	16	17	18	19	20	21	22	23	
July 14	11 A.M.	0	0	0	0	0	0	0	0	0	0	0	
" 15	11 A.M.	II	III	III		II	IV	I	III		II	III	
" 16	12 M.	IV	V	V		III	V		V		IV	IV	
" 17	4:30 P.M.	VI	VII			V	V		VII		VI	VI	
" 18	9 A.M.	VIII	VIII			VII			IX		VII		
" 19	10 A.M.												
" 20	11:30 A.M.												
" 21	9 P.M.												
" 22	9 P.M.												

The simultaneous study of the growth of the various colonies placed in apparently identical conditions, on the same slide or on adjacent slides, shows at first that the speed of growth is not the same for all the colonies. We have already seen that the interval between two divisions varies in rather large proportion, in the same stage, in two different colonies (*i.e.* four hours to seven hours between the division of *I* and that of *II;* ten hours to sixteen hours between the division of *II* and that of *III*).

Table I shows the records of twenty-three colonies (experiment commenced on the 14th of July); the figure 0 indicates the initial macrozooid at the beginning of the peduncle formation, and the Roman numbers indicate the number of the terminal individual on the main strain; we see, thus, that on the fourth day, there may be a difference of two generations between different colonies and that on the eighth day the difference may be four generations. The whole number of individuals borne by each colony differs, of course, proportionally.

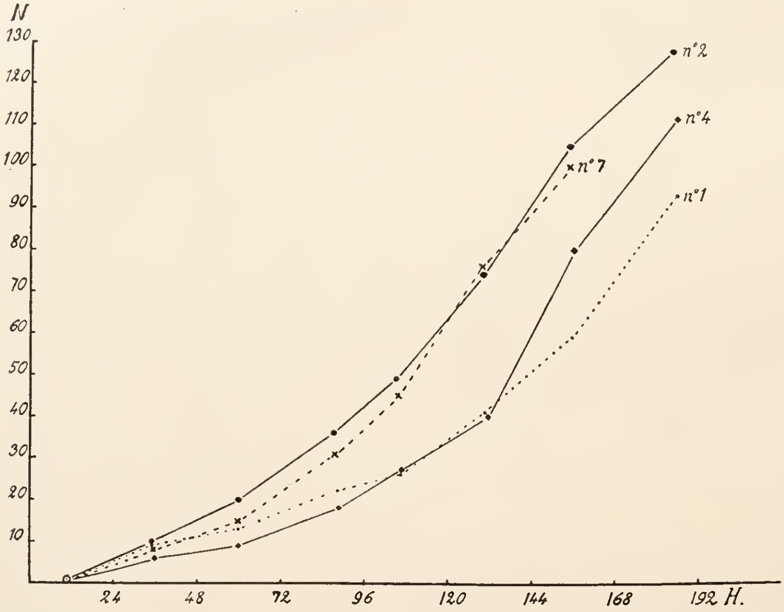

Fig. 16. Curves of growth from four colonies of *Z. alternans* (Nos. 1, 2, 4, and 7); number of the individuals in ordinates; time (in hours) in abscissæ.

The data relative to colonies Nos. 1, 2, 4, and 7 are plotted in the curve of Fig. 16. These are only gross numbers, there being no correction for some microzooids parasitised or dropped out. Besides, these various curves show that for each colony the rate of growth varies itself in the course of the growth; but it is difficult to determine the part of the accidental factors already mentioned and capable of introducing some disturbance.

Fig. 17 represents in function of time the genealogical and complete view of a colony having given sixteen generations on the main strain.

The essential data are given by the successive records of colony No. 2, completed, as regards the incomplete branches sprung from *A*, *B*, and *C*, by the data furnished by other colonies studied in the same experiment (3, 5, 20, etc.). Furthermore, the periods of some divisions have been settled according to the survey of the successive and periodical examinations of colony No. 2 with interpolations; I have kept account, in this case, of the interval settled with more precision than in other experiments in which either the first stages of the colony or the growth of a branch were connected at intervals of time most closely approached from hour to hour.

The curve represented in Fig. 18 is drawn according to this scheme. The daily increase of the number of individuals shows the following numbers:

Time (in hours)	Number of individuals	Increase of the unity of mass in 12 hours	Number of zooids made in 24 hours
0	1		1
12	2	2	
24	4	2	3
36	9	2.25	
48	15	1.66	11
60	23	1.58	
72	31	1.34	16
84	41	1.32	
96	55	1.34	24
108	66	1.20	
120	84	1.27	29
132	104	1.23	
144	122	1.17	38
156	137	1.12	
168	147	1.06	25

The first part of this tabulation shows a rather regular increase and such that the number of the individuals, *i.e.*, approximately the whole protoplasmic mass, doubles at regular intervals, from twelve hours to twelve hours.

Of course, we find again here, at first the geometrical progression of the ratio 2 which characterized the multiplication by bipartition of a mass of cells which keep always the same speed of growth. If we choose for unity of time this period of twelve hours, we see, however, that after the second day the rate of growth of the unity of mass, which averaged about 2, slows down progressively from 1.66 to 1.58 and 1.34, then persists for some time at a median and constant level: 1.32, 1.34, 1.20, 1.27.

Then, in a last period, this rate of growth again slows down with the

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values 1.23, 1.17, 1.12, 1.06; but the difficulty in obtaining an exact enumeration does not permit a determination of its values when the colony approaches its greatest size.

Then it appears that the growth progressively slackens in the whole of the colony; *the time necessary to double the protoplasmic mass grows as the protoplasmic mass increases;* it is a limiting factor of the growth.

But it is evident that this factor (or limiting factor), in the case of *Z. alternans,* is not a factor of senescence which affects equally all the individuals, and involves a sort of progressive segregation, whose nuclear phenomena give a parallel objective picture.

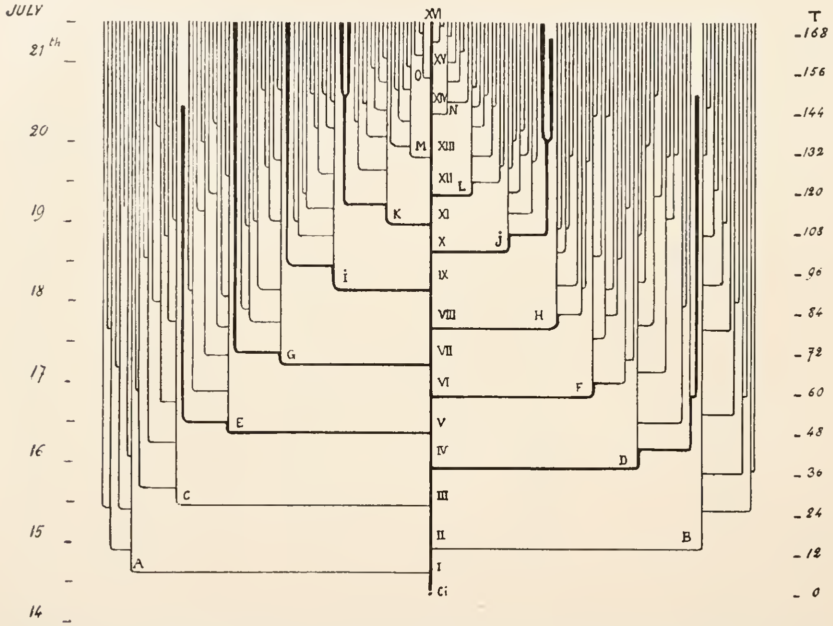

Fig. 17. Genealogical view of a colony at the sixteenth generation; time in abscissæ; lineage of each individual in ordinates.

This leads us to examine the case of the main strains. After the second division, which takes place rapidly, four to seven hours after the first one and at a temperature of 21° C., the rhythm of the bipartitions of the axial macrozooid slows down, (sixteen to seventeen hours between second and third divisions), then remains sensibly constant. During the entire growth of the colonies, more than twenty bipartitions of the axial macrozooid succeeded each other at intervals of ten to sixteen hours. The growth of the axial peduncle was fairly constant.

It seems then that during eight to ten days at least, the functional activity and the power of growth of the axial macrozooid remain constant, and, in the colonies already developed, one can observe the formation of a posterior ciliary crown around the terminal individual. Thus the axial macrozooid can become a migrating individual equivalent to a ciliospore, but one never observes in this case the endomictic transformation of the nuclear apparatus. We have seen how the nuclear segregation which is established during the differential divisions seems to determine the characteristic features of the median individuals and of the microzooids. However, we must admit that the later divisions of the microzooids are still different, although they are not accompanied by a visible nuclear segregation.

According to the rule of Claparède and Lachmann, we can still distinguish in one branch one main strain and lateral strains.

The fourth branch, for instance, after the differential divisions which separate $1D$ and $1d$ may be represented as follows: $1d$ gives $2d^2$ and $2d^1$. Let us give the exponent 1 to the main strain of this branch; $2d^2$ gives $2d^{21}$ and $2d^{22}$ which do not divide any further; $2d^1$ on the contrary gives $3d^2$ and $3d^1$. The smaller branch issued from $3d^2$ has a principal axis, but the number of generations is reduced. The first division separates $3d^{22}$, which does not divide any further, and $3d^{21}$, which gives $3d^{212}$ and $3d^{211}$ without descendants. The individual $3d^1$ gives $4d^1$ and $4d^2$; $4d^2$ gives $4d^{22}$ without descendants and $4d^{21}$, which still gives $4d^{212}$ and $4d^{211}$ without descendants. The individual $4d^1$

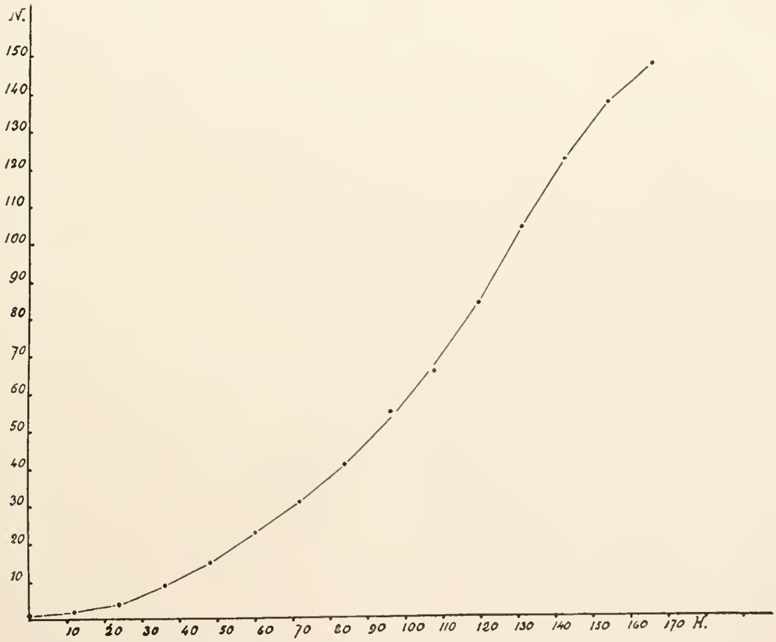

FIG. 18. Curve of growth of *Z. alternans* colony drawn from Fig. 17.

finally gives $5d^2$ without descendants, and $5d^1$ which divides into $6d^1$ and $6d^2$ without descendants.

The interval which separates the microzooid divisions is at first of the same order (or even more rapid) than the interval which separates the divisions of the axial individual; but it increases progressively and in such a colony, for example, the individuals of the sixth branch will represent six successive generations from the cell *F*, while its sister cell *VI* will have given during the same length of time ten successive generations.

We can see from Fig. 17, for instance, that the microzooids $2d^{22}$ and $2d^{21}$ live more than three days and a half without bipartition; such a fact is more typical with some microzooids of the earlier branches, *A* and *B*, which maintain themselves for more than five days without division. But after this time (corresponding to ten generations on the main strain of the branch) these individuals do not appear larger than the others; yet they feed and their protoplasm contains many digestive vacuoles. The decrease of the power of growth which characterizes these individuals is not dependent upon their age—and for this reason we cannot admit the notion of the factor of senescence—but of their position in the colony, as if the differential divisions assured the progressive segregation of a factor of growth. But we can still notice that this segregation, as it may be seen by the form of the growth of the branch *D*, for instance, is yet continued during the divisions of the microzooids which show no longer a differential appearance.

In short, if we bear in mind the main axis of the colony, its branches and its boughs, we see that the power of growth, and of multiplication, decreases according to a kind of gradient, in proportion with its removal from the main strain.

The differential character of the cellular divisions seems to be the essential condition which slows down and restrains the growth of the colonies of *Z. alternans*. But, theoretically at least, this restricted growth should go on indefinitely. It is not the case here. Secondary factors play here an important rôle; the development of different parasites (Protozoa, Protophytes) make it impossible to obtain a normal growth of the colonies beyond ten days, under ordinary laboratory conditions or in a natural marine environment; soon the last surviving individuals leave the common peduncle. The microzooids often form in this case a posterior ciliary wreath; their fate has not been determined.[3]

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[3] A few cases of conjugation have been observed between a terminal macrozooid and a migrating microzooid. These cases were rare; the later phenomena were not followed.

Conclusions

The sexual cycle described by Furssenko and by Wesenberg-Lund in the voluminous species of *Zoothamnium* (*Z. arbuscula* Ehrb., *Z. geniculatum* Ayrton) is rather special and with *Z. alternans* (Clap. and Lach.) I have never observed anything similar, either on the Brittany coast or in my cultures at Woods Hole; I will not, then, attempt to compare the evolutionary cycle of these different species. The objective that has led me into the minute study of these colonies of Vorticellidæ is the cyclical evolution—generally considered—of an initial cell's lineage, which is here the foundation macrozooid or the "ciliospore."

The growth of colonies of *Z. alternans* is limited, in a great measure, by external agents such as parasitic infections, or the growth of animal and vegetable microörganisms which change the surrounding conditions of a specific colony.

In the cultures watched as described above, these various circumstances, somewhat accidental, are much reduced; yet the growth of each colony appears to be limited *in itself;* I have taken the common individual—the microzooid—as unity of mass, and I have observed that the rate of growth decreases in function of time for the whole of each colony studied; at the same time, some particular migrating individuals are formed and become the source of new colonies; it is precisely this "cyclical" appearance of growth in the colonies of Vorticellidæ that I have described in an earlier paper (Fauré-Fremiet, 1922); I have considered two different hypotheses: (1) the formation during the evolution of the migrating individuals of a limited stock of an hypothetical "active substance" which divides and becomes increasingly smaller with each generation of daughter-cells, or (2) a progressive modification of the intimate composition of the cells, variations which would be "corrected" only during the evolution of their own migrating cells.[4]

In any case, this cyclical and limited evolution gives to the colonies of Vorticellidæ (*Epistylis, Carchesium, Zoothamnium*) somewhat of an individualized character. In this regard, the case of *Z. alternans* is very striking. At first, the successive divisions of the cells derived from the first individual and the regularity with which they follow one another in exactly determinate planes which fix the general features of the colony, closely recall the process of a strictly predetermined cleavage, but one which would be complicated with a continuous growth.

Secondly, the existence in these colonies of a main strain and of secondary strains characterized by different nuclear qualities and different evolutionary properties recall in a certain measure the separation of the germinative and somatic strains during the cleavage of an *Ascaris* egg.

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[4] These suppositions have been examined and criticized in a very interesting work of G. Teissier (1928).

Thirdly and finally, we can characterize the individuality of the colony by the repartition of the power of growth and the power of multiplication of its cells according to a certain gradient.

In connection with another species of *Zoothamnium* Wesenberg-Lund also considers the notion of the individuality of the colony, for the various individuals are tied by the continuous protoplasmic thread of the ramified peduncle and this brings about in their mass rather a physiological unity. But the above-indicated characteristics are again met, more or less accentuated, in other colonial Vorticellidæ in the species *Epistylis* and *Carchesium*, for example, which do not show any protoplasmic connection between the zooids.

The case of these colonies is then nearer that of a "population" of cells, and their cyclical evolution appears very similar to populations of free Infusoria, studied by so many authors.

The case of *Z. alternans* is still, from this point of view, particularly interesting. In these species, the Claparède and Lachmann rule shows that two daughter-cells have not necessarily the same power of growth and of proliferation. I found the same rule (1922) in some species of the genera *Carchesium* and *Epistylis,* and more especially with *Epistylis arenicolæ* (n. sp).

Here there seemed to exist in the course of the successive bipartitions a kind of progressive segregation of the power of growth, but we find in *Z. alternans,* as an objective support of this hypothesis, the differential divisions, which are produced at the origin of each lateral branch and which indicate a kind of nuclear segregation.

In this species the main strain's cells which keep a constant nuclear appearance, keep also a constant rate of growth and, apparently, an indefinite multiplicative power. We witness, then, a cytological mechanism, probably independent of the external factors which rule the functional differentiation of the cells belonging to the same family, in a process of growth.

This cytological factor, or those which are superimposed upon it, rules at the same time the family's general mode of growth; it intervenes as a limiting factor, independent of the colony's age, and quite distinct, by this fact, from a factor of senescence in the true meaning of this word. However, the colony's initial individuals, the "ciliospores," appear to be characterized by a kind of "physiological potential" greater than that of the main strain's common individuals.

As in all the colonial Vorticellidæ that I have previously studied, they are characterized by large size and by the presence of definite granulations connected with the secretion of the basic peduncle's inert substance.

During their particular growth, accompanied by a complete changing of the nuclear apparatus, the cells acquire these properties and we can thus show that near the end of the colony's cycle of growth an endomictic cycle exists, closely comparable to that observed in a population of free Infusoria.

But we must remark that, here again, the particular evolution of these "ciliospores" and the endomictic phenomena of which they are the seat, are determined, not by their *age*, but by their place in the colony's plan, just as if this evolution were still connected with the same mechanism of differential division and of nuclear segregation.[5]

I am very glad to be able here to express my thanks to the International Education Board, to my American colleagues who made my residence at Woods Hole so profitable for me, and, very particularly, to Dr. Calkins and Mrs. Harnley, who have helped me in translating this paper.

SUMMARY

1. The first division of the initial macrozooid (or ciliospore) determines the median antero-posterior plane of the colony; the subsequent cleavages of the daughter individuals are brought about according to equally determined schemes, which give the main strain (or axial trunk) and the lateral branches, alternately at right and at left.

2. The individuals constituting the main strain are of a rather large size (axial macrozooids); their cleavage is always accompanied by a differential division giving rise to a new axial macrozooid and a median microzooid.

3. The differential divisions are characterized by an unequal division of the protoplasmic mass, accompanied either by a sensibly equal division of the macronucleus (division supposed to be quantitatively differential), or by the unequal division of the macronucleus in which the larger mass (delicately granular) remains in the larger individual, while the thinner part (often of fibrillar structure) goes to the microzooid (division supposed to be qualitatively differential).

4. The cleavages of the ciliospores and those of the axial macrozooids, I, II, and III are always differential as regards the protoplasm and the nucleus. The cleavages of the macrozooids IV and after give a cytoplasmic differential division and an equal nuclear division; the dif-

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[5] Long ago I mentioned an apparently differential division in *Lagenophrys*, in which one of the individuals remained sedentary, while the other migrated and secreted a new shell (1904).

ferential division of the macronucleus is carried back to the cleavage of the corresponding median microzooids.

5. The common microzooids have a limited power of growth and of multiplication.

6. The median individuals having a large macronucleus after the differential division of the median microzooids *D* and progeny begin an active period of growth accompanied or unaccompanied by only one ulterior division: these forms constitute the median macrozooids or "ciliospores."

7. The growth of the ciliospores is accompanied by an important hypertrophy of the macronucleus followed at first by a disintegration, then by a reconstitution through an endomictic process.

8. During the growth of the median macrozooids, some grains of secretion accumulate at the individual's posterior end, then the ciliary crown grows, the ciliospore breaks away, swims freely, then settles down on a substratum and becomes the source of a new colony.

9. The character of the differential divisions on the main strain seems to determine the individual's differentiation of the colony; this differentiation depends not only on the individual's size, but also on its physiological potencies.

10. Independently of the obviously differential divisions, it is shown that the power of growth is divided among the microzooids according to a gradient, so to speak.

11. The unequal power of growth of the various individuals of a colony gives to its whole growth a behavior which approaches the behavior of an organism. This unequal share constitutes for the growth of the whole a limiting factor very unlike a factor of senescence.

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THE INFLUENCE OF HUMIDITY ON THE BODY TEMPERATURE OF CERTAIN POIKILOTHERMS

F. G. HALL AND R. W. ROOT

(From the Zoölogical Laboratory, Duke University)

Poikilothermic animals are commonly accredited with possession of a body temperature closely approximating that of their environment. In general this appears to be true. However, there are some cases where the body temperature of certain " cold blooded " animals may be very unlike that of their surroundings. Such examples are given by Rogers and Lewis (1916) in a table which they have compiled from the investigations of numerous workers. It shows that not all investigators agree even as to the temperature of the same species. It is probable that much of the discrepancy is due to different types of method. On the other hand, a more careful examination of the conflicting results of various authors as to the correspondence between body and environmental temperature shows that the greatest variations occur when animals are subjected to atmospheric conditions.

The factors which influence the temperature of animals may be classified as follows: *intrinsic*—those that lie within the organism and act to produce a temperature different from that of the environment; *extrinsic*—those imposed on the animal from without. The extrinsic factors are (1) conduction and convection, (2) radiation, (3) evaporation of water. A discussion of the rôle played by each factor is given by Pearse and Hall (1928). It is the purpose of this paper to study the influence of the third factor, namely, the evaporation of water, on the body temperature of various poikilotherms.

EXPERIMENTAL METHODS

Apparatus.—The apparatus employed consisted of an air pump, several gas washing bottles—some containing concentrated sulfuric acid, others water—a chamber in which animals under experimentation were placed, temperature-measuring instruments, which included a potentiometer, a high sensitivity suspension galvanometer, and a copper-constantin thermocouple.

The air pump was adjusted to supply air at a constant rate of 22.6 liters per minute through two possible air leads. One lead was through four wash bottles containing concentrated sulfuric acid and the other through four similar wash bottles containing pure water. The amount of air passing proportionally through each lead was controlled by screw pinch cocks. Thus air of any desired humidity from 7 per cent to 100 per cent could be obtained. The relative humidity of the air was measured by a calibrated hair hygrometer suspended in an enclosed jar through which all the air passed before entering the experimental animal chamber.

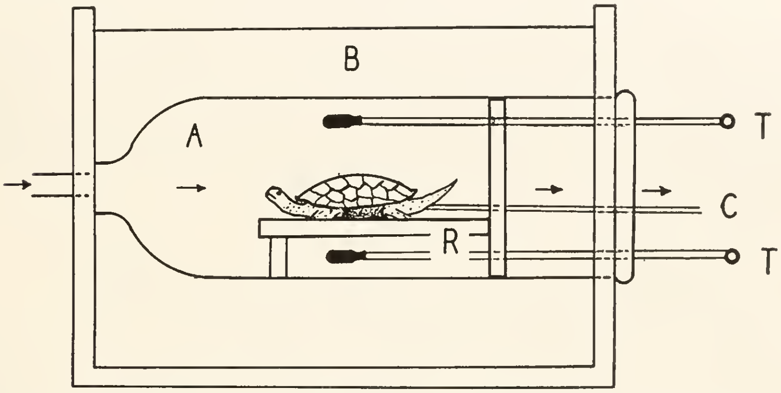

Fig. 1. Apparatus used to determine the influence of relative humidity on the body temperature of animals.

The experimental chamber in which animals were placed is shown in Fig. 1. A cylindrical percolator (*A*) was immersed in a constant temperature bath (*B*). Animals were tied to a sliding rack (*R*) which was so arranged that only a small portion of the animals' bodies was in contact with it, thus allowing a maximum surface to be exposed to the moving air. The end of this rack closed the mouth of the percolator. Two precision thermometers (*T*) were inserted through the rack, one in the upper, the other in the lower portion of the percolator. The thermocouple lead wires (*C*) also passed through the end of the rack. The mouth of the percolator was packed with cotton to lower the rate of conduction. The direction of air flow is shown by arrows. The temperature in all experiments was maintained at 20° C.

Experimental Animals.—The species chosen for this investigation were: Amphibians—the frog, *Rana pipiens* Schreber; the salamander, *Plethedon glutinosus* Green; the toad, *Bufo fowleri* Garman. Reptiles—the lizard, *Sceloporus undulatus* Latreille; the "horned toad," *Phrynosoma cornutum* Harlan; the turtles, *Terrapene carolina carolina* Linn., *Cistudo major* Agassiz, *Chrysemys marginata* Agassiz; the alligator, *Alligator mississippiensis* Daudin. All animals were kept under good laboratory conditions, and were alive and active at the end of each experiment. Individuals were weighed at the beginning and end of each experiment. From four to ten individuals of each species were used and several determinations were made on each individual.

Temperature Records.—Environmental temperatures were recorded by use of precision thermometers placed in the experimental chamber. The body temperature was determined with a thermocouple inserted through the anus well up into the animal's body. Each thermocouple used was calibrated against a precision thermometer (previously calibrated by the U. S. Bureau of Standards). The temperature readings are believed to be accurate to ± 0.01° C. Records of the temperature of each animal and its environment were made at the following relative humidity points: 7 per cent, 25 per cent, 50 per cent, 75 per cent and 95–100 per cent.

TABLE I

Showing Variations in Body Temperature of Several Species of Poikilotherms from Environmental Temperatures in Atmospheres of Different Relative Humidities

Species	Relative Humidity				
	7	25	50	75	95–100
Salamander	−9.21	−6.34	−4.62	−2.54	−0.29
Frog	−8.60	−6.75	−4.68	−3.01	−0.13
Toad	−7.33	−5.31	−3.98	−2.48	−0.74
Lizard	−0.70	−0.70	−0.15	+0.30	+0.64
Horned "Toad"	−0.37	+0.02	+0.11	+0.19	+0.38
Turtle, water	−0.72	−0.57	−0.52	−0.41	−0.12
Turtle, land	−0.34	−0.23	−0.11	−0.03	+0.15
Alligator	−0.39	−0.26	−0.15	−0.08	+0.18

Plus signs signify a higher body temperature than that of the environment; minus signs indicate a depression in body temperature below that of the environment.

RESULTS

Amphibians.—The body temperature of the salamander, frog, and toad very closely approximated that of their environment when the surrounding atmosphere was saturated, or nearly so, with water vapor. In atmospheres of low humidity, however, a considerable depression in the body temperature below that of the environment was obtained. Salamanders showed the most marked depression, toads the least marked. The average results obtained are shown in Table I. Considerable weight loss was suffered by these animals. At low humidities their skins appeared dry and their bodies emaciated.

Reptiles.—The response of reptiles to atmospheres of varying humidity was quite unlike that of amphibians. Whereas amphibians showed great depression in body temperature when exposed to a dry environment, reptiles showed only slight depression. In fact, if the relative humidity be maintained between 90 and 100 per cent, many reptiles will show a body temperature slightly higher than that of their surroundings. Lizards and water turtles (*Chrysemys marginata*) were influenced the most by low humidity. Apparently the water turtle is slightly more susceptible to the influence of humidity than the land form. Weight loss in the reptiles was practically nil. Subjection to low hu-

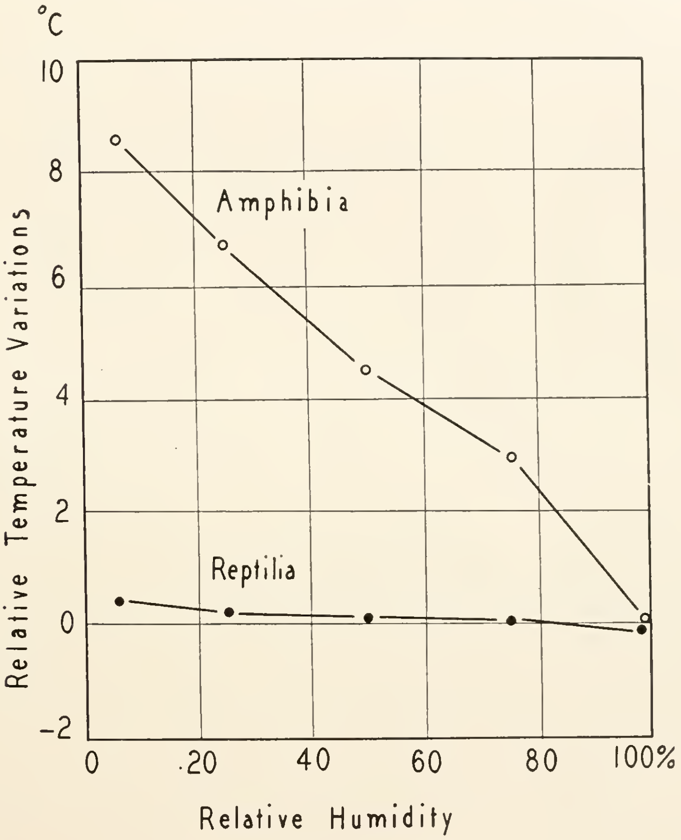

FIG. 2. Graph showing relation of body temperature to environmental temperature of amphibians and reptiles when subjected to different relative humidities.

midity for long periods of time showed no apparent injurious effect. Table I contains the average results obtained on all forms summarized to show the difference in response to surroundings of varying relative humidity. Fig. 2 shows the comparison of the response of amphibians as a group with that of reptiles, and shows the variation in the change of body temperature from that of the environment at similar relative humidities.

DISCUSSION

It is apparent from the results obtained that in atmospheres of low relative humidity, amphibians will have a much lower body temperature than that of their environment. Such a condition results from the evaporation of water from the surface of the body. The body temperature of reptiles is but slightly changed by similar conditions. Thus it is clearly indicated that the difference in response of these two classes lies in the type of integument. The amphibians with moist skin will readily lose water by evaporation. They have little means of retaining water as has been shown by Gray (1928). The moisture of their integument is in dynamic equilibrium with the water content of their environment. The inner tissues supply water when that at the surface has been evaporated (Hall, 1922). Thus, for example, a salamander behaves physically very much like a wet bulb thermometer. The depression in temperature is not as great, probably because water is not transported to the surface as rapidly as in the wick of a wet bulb thermometer.

Amphibians are limited in their habitat to moist places. They possess a "reaction pattern" (Pearse, 1922), which permits them to live only under damp logs and stones or in marshes or other watery places. Thus they become more conspicuous on rainy days when the atmosphere offers a more favorable and less restricted environment for their activities. It is perhaps interesting to speculate that a frog may have a lower body temperature on a dry, sunny day than on a somewhat colder, rainy day.

The possession of a scaled integument, characteristic of the reptiles, greatly increases the power of water retention. Reptiles give up water very slowly and will resist desiccation for long periods of time (Hall, 1922). Not only by possession of an integument, but by certain internal physiological processes, such as the elimination of nitrogenous wastes as uric acid instead of urea, they conserve water. In consequence many reptiles live in very dry surroundings.

Perhaps the principal explanation of the discrepancies in reports by many investigators of the correspondence between body and environmental temperatures is that they are due to a lack of control or record of humidity. In the light of these experiments any results obtained without knowledge of the relative humidity of the surroundings in which an animal's temperature is taken would seem meaningless.

A further observation seems to indicate that the influence of changes in humidity on the body temperature of these animals decreases as animals higher in the phylogenetic series are used. It appears that amphibians as they progress in evolution show a decrease in their susceptibility to humidity variations. The same fact apparently holds for the reptiles. Fig. 3 represents the results arranged to show the maximum change in body temperature relative to environmental temperature in each of the species used, the salamander showing the greatest change, the alligator the least. The reptiles seem to have a more stable body temperature than amphibians because they are less influenced by environmental factors. Possibly the increased ability of water retention evolved in the reptiles is a " milestone " on the road to homoiothermism.

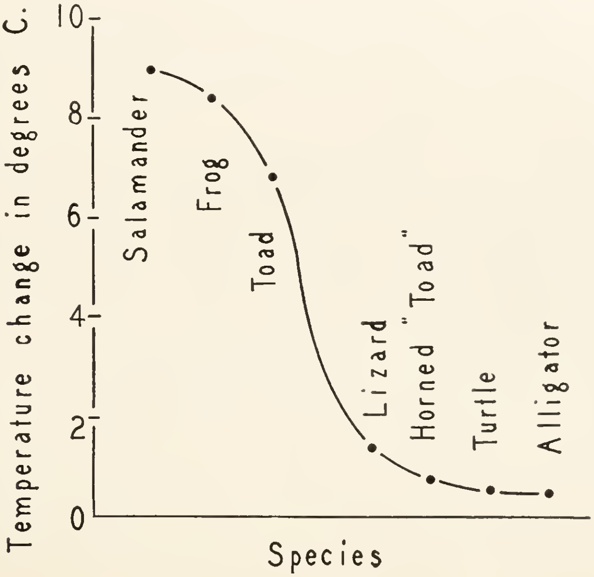

FIG. 3. Showing a comparison of the change in temperature of the body of each species studied when the humidity was lowered from 100 per cent to 7 per cent saturation.

SUMMARY

1. Amphibians show marked response in body temperature to environmental variations in relative humidity. When subjected to an atmosphere of 7 per cent relative humidity at 20° C., a depression of several degrees centigrade may occur in their body temperature.

2. Reptiles show very little response to variations in relative humidity. The integument apparently prevents the evaporation of moisture from the surface of the body.

3. It is suggested that the evolution of the scaly integument of reptiles from the slimy and moist skin of amphibians, with the concomitant power of water retention, is perhaps an important step in the evolution of homoiothermism.

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THE POINT OF ENTRANCE OF THE SPERMATOZOÖN IN RELATION TO THE ORIENTATION OF THE EMBRYO IN EGGS WITH SPIRAL CLEAVAGE

T. H. MORGAN AND ALBERT TYLER

(*From the Marine Biological Laboratory, Woods Hole, and the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology*)

If the entrance of the spermatozoön into the egg is instrumental in determining the planes of cleavage, and the cleavage planes bear a definite relation to the embryonic axes, it would still remain important to find out whether the side of the egg on which the sperm enters is a factor in locating the dorsal (or ventral) side of the embryo. In some eggs having an equal first cleavage, such as the frog, the ascidian and the sea-urchin, observations of this kind have been reported, and a distinct relation has been found between the side of the egg on which the sperm enters and the future dorso-ventral axis of the embryo. Curiously enough, despite the large number of careful observations on the cell-lineage of eggs with a spiral type of cleavage, there is only one set of observations on the relation of the entering point to the first cleavage plane, and even here we do not know whether the side on which the sperm enters becomes the dorsal or the ventral side.

In the course of our work another relation was found that is both novel and has a bearing on the interpretation of the so-called law of alternate right- and left-cleavage in spiral types. In *Cumingia* it was discovered that two types of second cleavage occur in equal numbers, one of which in ordinary parlance would be called a right-handed, the other a left-handed spiral, yet in both cases the third cleavage was found to be always dexiotropic. As a consequence of this relation it follows that in one case the first plane of cleavage corresponds to the median plane of the embryo, and in the other case the second plane of cleavage corresponds to the median plane, provided the later sequence of events is the same for both types.

A third relation has not, so far as we know, been carefully studied, namely, whether in eggs with an unequal first cleavage, the plane of cleavage passes through the pole or consistently to the side. Without exception our observations show that the plane passes to the side on which the smaller cell comes to lie, but the relations here are not the same in the three types examined, nor are the succeeding events always the same. However, these relations will be shown to have a significant bearing on the location of the median plane of the body.

The Cleavage of Cumingia

The early cleavage of the egg of the bivalve mollusk *Cumingia tellinoides* has been described by Morgan (1910) and Browne (1910). The following observations were made in the summer of 1929 at Woods Hole, Mass. The eggs and sperm were obtained by the usual method of isolating individuals in small dishes of sea water. The eggs were washed and samples removed for fertilization at once or soon after deposition. A square of vaseline was laid down on a slide and two fragments of No. 2 cover slips placed on the vaseline for additional support. A drop of eggs was placed in the square and a small drop of very dilute sperm-suspension was added. A cover slip was placed on the preparation and the slide was examined at once under the microscope. The eggs were brought under observation in less than thirty seconds after insemination. To some of the eggs one or more spermatozoa were already attached; to others they soon became attached. Only those cases in which one or a few spermatozoa were attached were followed—if the insemination had been too heavy the slide was rejected. The egg of *Cumingia* is about 66 micra in diameter, and with the jelly about 107 micra. The glass supports were about 140 micra in thickness, which with the further help of the vaseline sufficed to prevent compression of the eggs.

The pole of the egg of *Cumingia* can readily be identified by a clear area free from pigment. The outer pole of the first maturation spindle lies in the center of this area. The identification of the pole is later checked by the point of extrusion of the polar bodies. The sperm enters at any point of the periphery of the egg. On attaching itself to the egg the spermatozoön becomes immotile, its tail extending radially from the surface. About 30 seconds after attachment the egg rather suddenly becomes distinctly ovoid in shape, with the more pointed end at the point of attachment. This change in shape lasts 30 seconds or less. As the egg rounds out again the sperm enters. This phenomenon enables one to identify the particular sperm that will enter, even before the sperm-head has penetrated. Other sperms in the jelly, apparently even touching the surface of the egg, do not call forth this striking reaction. The change in shape is something more than the formation of a fertilization cone, since it involves a change in form of the whole egg. Unless the entering sperm is exactly on the horizon, the change in form of the egg may not be observed; also there seems to be some difference in different sets of eggs as to its appearance.

The first polar body appears five or six minutes after fertilization. In making observations, all the sperms at or near the periphery of the egg were located on a drawing, and their relative position in three dimensions noted. Those that did not enter served as markers. Spermatozoa that are too far above or below the optical section of the egg cannot always be seen. When the polar body appears, the pole can be more accurately located in relation to the position of the entrance point. As a rule only one egg in each preparation was followed. The observations were made under magnifications of 284 and 440 diameters.

The first cleavage appeared about 50 minutes after fertilization. The location of the plane was noted in the drawing with respect to the point of entrance. This was checked as far as possible by the position of the markers, since, if any shifting of the egg occurred, their positions would change. The first division; Fig. 1, *a, b,* is unequal.

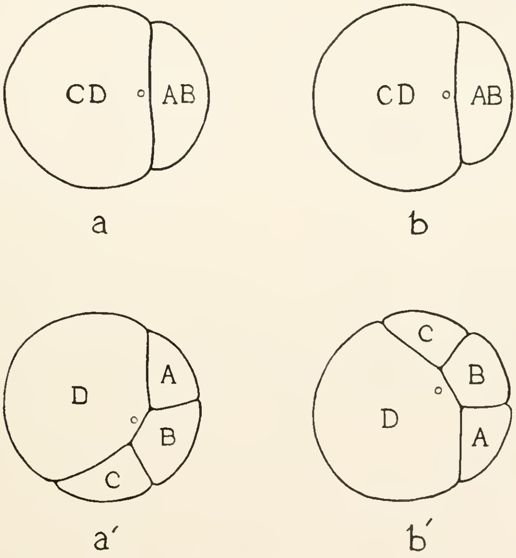

FIG. 1. The first and second cleavages of *Cumingia* showing the two possible types of 4-cell stage. In *a′* the *C*-cell comes off "counterclockwise"; in *b′* "clockwise."

The smaller blastomere, following the convention for this type of egg, will be called *AB,* and the larger blastomere *CD.* The second cleavage, Fig. 1, *a′*, *b′*, divides *AB* equally (*A* and *B*), and *CD* into unequal parts (*C* and *D*); the *C*-blastomere being smaller and approximately the size of *A* or *B*. Theoretically the *C*-cell might form from either side of *CD* (Fig. 1, *a′*, *b′*). It is obvious, then, that there would be two possible configurations or arrangements of the blastomeres after the division that are mirror figures of each other (Fig. 1, *a′*, and Fig. 1, *b′*). As will be shown, it is important at this stage not to identify these two types as dextral or sinistral cleavages, although this would be the usual interpretation.

The clockwise sequence *ABCD* may seem to imply that the second cleavage has been leiotropic and the third will be dexiotropic, or conversely for the counter-clockwise sequence *DCBA*; but by utilizing the usual lettering we do not wish here to commit ourselves to such an implication. The reasons for this will appear later.

Entrance Point of Spermatozoön in Relation to the First Cleavage in Cumingia

Ninety-eight cases were recorded in which the relation of the entrance point to the first cleavage plane was definitely ascertained. In 77 cases there was strict coincidence between the plane of the first division and the entrance point. In 13 the entrance point was less than 45° from the cleavage plane. In 8 cases the divergence was greater than 45° and less than 90°. Whether the expectation of close coincidence should be 100 per cent and the departures be considered as due to abnormalities, or as due to errors of observation may be briefly considered.

Polyspermy might introduce a complication, but it can be detected either by the presence of extra pronuclei, or by irregularities in the cleavage. Compression of the egg might be one of the factors determining the position of the cleavage plane. To avoid this, the supports were made so thick that the space between the slide and the cover slip was greater than the diameter of the egg plus the jelly. If the sea water evaporates, the retreating edge of water may cause the egg to move, and the hypertonicity might cause irregularities in cleavage. This was avoided to a large extent by the wall of vaseline; also eggs were selected that lay in the centre of the drop. Any movement of the eggs can be detected by their position with respect to neighboring eggs. The change in shape that the egg undergoes before cleavage is not a serious source of error, especially if checked by the presence of "markers" on the egg, but during division the change in shape of the egg may cause slight changes in position. Therefore, whenever possible, the egg was constantly watched throughout this period. In some cases when the cleavage is horizontal the egg may roll over. This is prevented to some extent by avoiding jarring of the table etc. When one or more of these factors was observed to come into play, the egg under observation was rejected.

The first cleavage plane does not pass through the pole (as determined by the position of the attached polar bodies), but slightly to one side. When considered from the entrance point of the sperm, the pole of the egg being up, this plane may be said to pass to the right or to the left of the pole. Whenever the first plane passes to the right of the pole, the *AB*-cell comes to lie to the right of the entrance point (Fig. 2, *a*); whenever it passes to the left of the pole, the *AB* comes to lie to the left (Fig. 3, *a*). This simple relation, which is constant in all the eggs examined, has apparently been overlooked by earlier observers in eggs of this type. The polar bodies adhere to the surface of the *CD* blastomere, and are carried into the furrow during the first division. Of the 77 cases of coincidence between the entrance point and the first plane, the *AB* was to the right in 40 cases and to the left in 37 cases. It appears that the chances are equal that the smaller cell lies to the right or to the left of the entrance point. The bearing of these two possibilities on the location of the plane of bilateral symmetry will be considered presently.

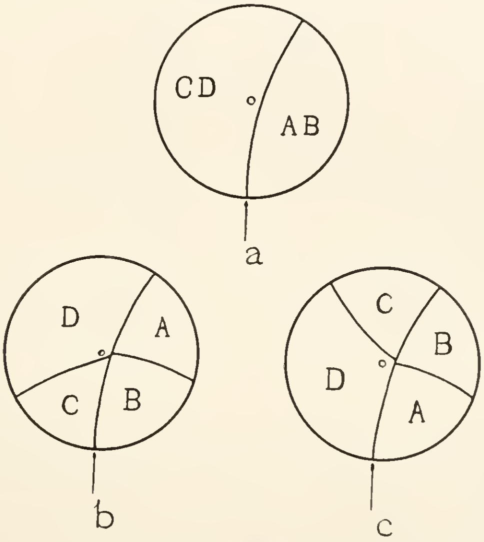

FIG. 2. The cleavage planes of *Cumingia* with respect to the entrance point of the spermatozoön. *a*, 2-cell stage with *AB* to right of sperm-entrance point; *b* and *c*, the two possible types of second cleavage.

It is obvious that when the small cell (*AB*) lies to the right of the entrance point there are two possible types of second cleavage (Fig.

2, *b,* and Fig. 2, *c*); similarly when the small cell (*AB*) lies to the left (Figs. 3, *b*, and 3, *c*). As a matter of fact it was found in these 77 cases of coincidence that when the *AB* was to the right, only one of the two theoretical types appeared, namely, that shown in Fig. 2, *b*. When the *AB* was to the left, again only one of the two theoretical types appeared, namely, that shown in Fig. 3, *b*. Ordinarily the cleavage giving the first type (Fig. 2, *b*) would be called a leiotropic second cleavage, implying that the third would be dexiotropic. The second type, Fig. 3, *b,* would be called a dexiotropic second cleavage, implying a leiotropic third. However, a study of the third cleavage of *Cumingia* has shown that the division is always dexiotropic. This information was obtained from eggs preserved at the time of the oncoming third cleavage. The orientation of the spindles with respect to the poles was determined in 84 eggs, and in every case they showed the cleavage to be dexiotropic (Fig. 5). The observation shows in the first place that it would have been erroneous to conclude that because the third cleavage is dexiotropic, the second must have been leiotropic. It would have been equally erroneous to have concluded from the two types of four-cell stages that the direction of the spiral would be different in the two types. By parity of reasoning it would seem unjustifiable to infer that because a given egg shows a leiotropic second cleavage, the first cleavage must have been dexiotropic, and thus to designate the egg as a dexiotropic egg.

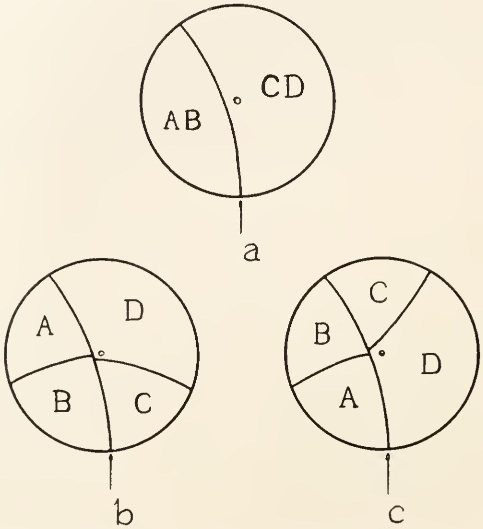

FIG. 3. The same as Fig. 2, except that the *AB*-cell lies to the left of the sperm-entrance point.

Such reasoning might have led one to infer that a dexiotropic third cleavage in *Cumingia* means that the second cleavage must have been leiotropic. A study of preserved eggs in the anaphase of the second division gave no indication of a spiral arrangement of the spindle. The spindles in the *CD*- and *AB*-cells appear to lie in the same horizontal plane (Fig. 4, *a*, *b*, *c*, *d*), instead of being tilted in opposite directions, as has been described for other eggs at this division (Mead, Conklin). Of course it is possible that the tilting of the spindles in the *Cumingia* egg is too slight to be visible, but nevertheless it is interesting to note that in this egg in which two different types of four-cell stages occur the spindles do not show a visible tilting. The spindles, in the *AB*- and *CD*-cells, are horizontal as shown in the figures (Fig. 4, *a*, *b*, *c*, *d*). However, they are not parallel, but, especially in the *CD*-cell, the spindle makes an angle with the plane of division.

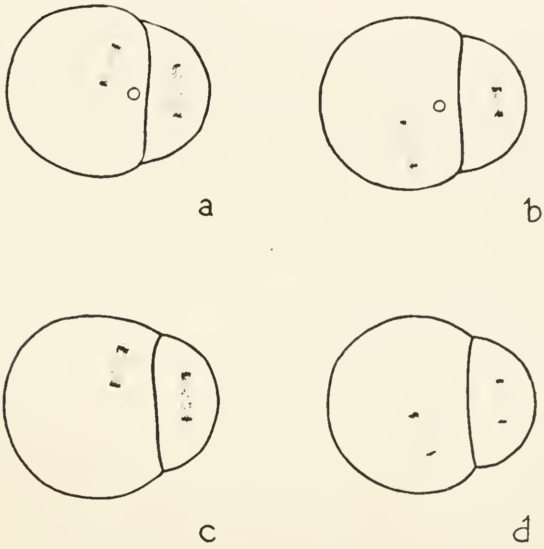

Fig. 4. Two-cell stages of *Cumingia* showing the positions of the spindle for the second cleavage. *a* and *b*, polar views; in *a* the *C*-cell will come off clockwise, in *b* the *C*-cell will come off counterclockwise. *c* and *d*, antipolar views; in *c* the *C*-cell will come off counterclockwise, in *d*, clockwise. The two poles of the spindles appear to lie at the same level in all cases.

In order to answer the question, if it should arise, as to whether both types of cleavage in *Cumingia* produce normal embryos, a few eggs of each type were isolated. Normal embryos developed from each.

The normal trochophore swims in a dexiotropic spiral. This also occurred in the embryos from these two types. Moreover, *all* the embryos from a culture swim in the same kind of spiral. In adult *Cumingia* the two valves of the shell are different in the articulation joint on the median dorsal side. All shells examined were alike, *i.e.*, not right or left, but all the same.

Location of the D-Cell in Relation to the Entrance Point

It has been found that when the first plane passes to the right of the pole (Fig. 2, *a*) the next division is always of such a sort that the *D*-cell is later away from the point of entrance of the sperm (Fig. 2, *b*). Similarly when the first plane passes to the left of the pole (Fig. 3, *a*) the next division is always of such a sort that the future *D*-cell is again away from the point of entrance (Fig. 3, *b*). The records from living eggs show that in 32 cases in which the cleavage plane passed to the right of the pole, the *D*-cell lay on the side opposite the entrance point, giving the arrangement of the blastomeres shown in Fig. 2, *b*. In 30 cases in which the plane passed to the left, the *D*-cell also lay on the side opposite the entrance point, as in Fig. 3, *b*. No exceptions to this rule are found.

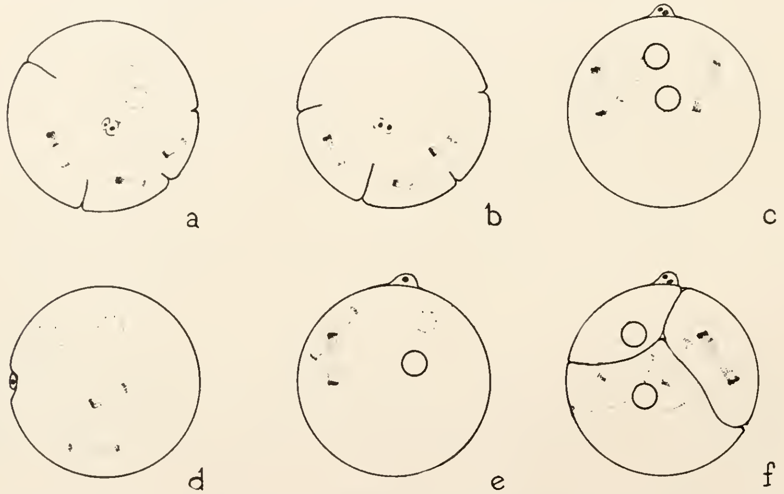

FIG. 5. Four-cell stages of *Cumingia* showing the position of the spindles for the next division. In all cases the spindles show that the next division will be dexiotropic.

So far the description has been restricted to those cases where the first cleavage plane coincided very nearly with the entrance point. In addition there were a few other cases, as reported above, where the coincidence was not so close and where there were no reasons to suppose that errors of observation were made. There were 13 such cases recorded in which the cleavage plane was less than 45° from the entrance point. If the entrance point is arbitrarily brought to the nearest point of the actual cleavage plane, then there are 3 cases in which *AB* is to the right of the entrance point, and 10 cases in which *AB* is to the left. The same relations of *D*-cell to entrance point obtain for both of these sets of cases as for those in which there was strict coincidence. There were also 8 cases in which the first cleavage plane was more than 45° from the entrance point. This divergence is too great to make a comparison profitable.

Relation of the Entrance Point of the Sperm to the Plane of Bilateral Symmetry

The evidence reported above has an important bearing on the relation of the point of entrance of the sperm to the plane of bilateral symmetry of the body. It has been shown that in 78 per cent of the cases close coincidence was observed between entrance point and first cleavage plane. In about half of these the first cleavage passed to the right of the pole (Fig. 2 *a*), giving the type of 4-cell stage shown in Fig. 2, *b*. At the next cleavage, the third, the 1-*d* micromere forms dexiotropically (Fig. 5). If from this point onwards the cleavages alternate, left and right, the 4-*d* cell will come off leiotropically and will lie next to the second plane of cleavage as shown in Fig. 6, *a*. It has been shown (Lillie, 1895) for at least one pelecypod (*Unio*) that the 4-*d* blastomere gives rise to the larval mesoblast, and establishes the plane of bilateral symmetry. This means that the second plane of cleavage coincides approximately with the median plane of the body.

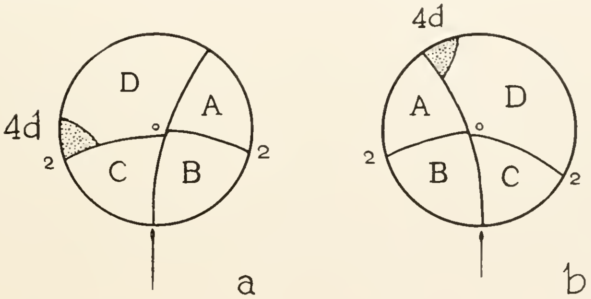

FIG. 6. Diagrams indicating the location of the 4-*d* cells in the two types of cleavage shown in Fig. 2, *b*, and in Fig. 3, *b*.

In the other half of the recorded cases the first cleavage passed to the left of the pole (Fig. 3, *a*) giving the type of 4-cell stage shown

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in Fig. 3, *b*. The 1-*d* again forms dexiotropically, Fig. 5. It follows from the same reasoning that the 4-*d* micromere comes off leiotropically, and will here lie next to the first plane of cleavage as shown in Fig. 6, *b*, and this plane of cleavage will now approximate the median plane of the body.

It may seem, then, that either the first or the second plane of cleavage may become the median plane of the body. This follows only on the assumption made above, which, although known to be true for other eggs, has not been entirely shown in this case. It is possible, for example, that the second somatoblast which determines the median plane may be formed at different divisions in the two cases. If, for example, in the type shown in Figs. 3, *a*, and 3, *b*, the second somatoblast appeared one division earlier or one division later, the median plane would be the same as in the other case (Fig. 2, *a*, and 2, *b*). As shown by the evidence, when the first cleavage plane passes to the right of the pole, the plane of bilateral symmetry coincides with the second cleavage plane, and when it passes to the left, with the first cleavage plane. What determines the passage of the first cleavage plane to the right of the pole in some cases and to the left in others is unknown. The fact that about 50 per cent of each type occurs suggests that it is merely a matter of chance. If we assume that the unfertilized egg has its materials radially arranged around the polar axis, and that the entering sperm determines through movements of the contents of the egg (or otherwise) that materials correlated with the determination of the *D*-cell come to lie opposite the entrance point of the sperm; and furthermore, that the cleavage plane does not pass through this material, then a possible interpretation suggests itself. It is obviously not necessary to make this assumption in quite the same crude form as suggested above in order to express these relations, for, at the time of the first division, all of the egg appears to be involved in the process. The risk of making such a generalization will be apparent when another egg, *Chætopterus,* is examined.

The Cleavage of Chætopterus

The eggs were washed in sea water, and allowed to stand about 20 to 30 minutes during which time the first polar spindle forms. A drop of eggs was put onto a slide prepared in the same way as for *Cumingia.* The egg measures 106 micra in diameter, without the jelly, and 111 micra with the jelly. The same thickness of cover slip support etc. was used as for *Cumingia.* A very small drop of very dilute sperm-suspension was added to the eggs which were examined immediately. In most cases the spermatozoa were already attached as though the combination had been made almost instantaneously. The spermatozoön enters 15 to 30 seconds after insemination and may be missed unless the preparation is examined very quickly. The pole of the egg can be identified by the clear area in which the spindle for the first maturation division lies. The sperm enters at any point, and a slight fertilization cone appears at the point of entrance. The extra sperm which do not enter remain attached, and serve as markers. The exact position of the pole is given by the location of the polar bodies.

The cleavage of the egg of *Chætopterus* has been described by Mead, Wilson, and Lillie, and the relation of the median plane of the body to the first cleavage plane determined, but so far no one has examined the relation of the entrance point of the sperm to the first cleavage. The third cleavage of the egg is dexiotropic, and the fourth leiotropic, so that 2-*d* (the first somatoblast) comes off near the second cleavage plane, and 4-*d* (the second somatoblast) is similarly placed. This determines that the median plane of the body lies near the second cleavage plane.

The Relation of the Entrance Point of the Sperm to the First Cleavage Plane

As in *Cumingia* the location of the sperm that had entered was recorded on the drawing, and the individual eggs watched until the cleavage furrow appeared. In 48 eggs there was a fairly strict coincidence; in 35 eggs the entrance point was less than 45° from the plane of the first division, and both to the right and left of the plane. In 33 eggs it was more than 45° and less than 90° to the right and left. Thus in only 41 per cent of the cases was there a close agreement between entrance point and cleavage plane; but if the entrance point is not in some way correlated with the direction of the first cleavage plane, even this percentage of coincidence would not be expected. Taking first the cases where coincidence occurs, it was found that in 23 cases the first plane passed to the right of the pole, which means that the *AB*-cell lay to the right of the entrance point as in Fig. 7, *a*. In 25 cases it passed to the left of the pole, thus placing the *AB*-cell to the left of the entrance point as in Fig. 7, *b*. In both cases, however, the second cleavage gave the same arrangement of cells, namely, that shown in Figs. 7, *a*′, *b*′. According to the usual convention these four-cell stages would be obtained from leiotropic second cleavages (which is actually true for the *Chætopterus* egg), but in one type, Fig. 7, *a*′, the *D*-cell would lie away from the entrance point of the sperm, and in the other type near the entrance point (Fig. 7, *b*′).

The third cleavage in all cases observed, both in the living and in the preserved eggs, was dexiotropic. If the subsequent cleavages alternate left and right, the 4-*d* cell in both types will come to lie near to the second plane of cleavage (Fig. 7, *a*′, *b*′). This means that the second plane coincides with the median plane of the body, although in one type the entrance point of the sperm would be to the right of the median plane, and in the other it would be to the left.

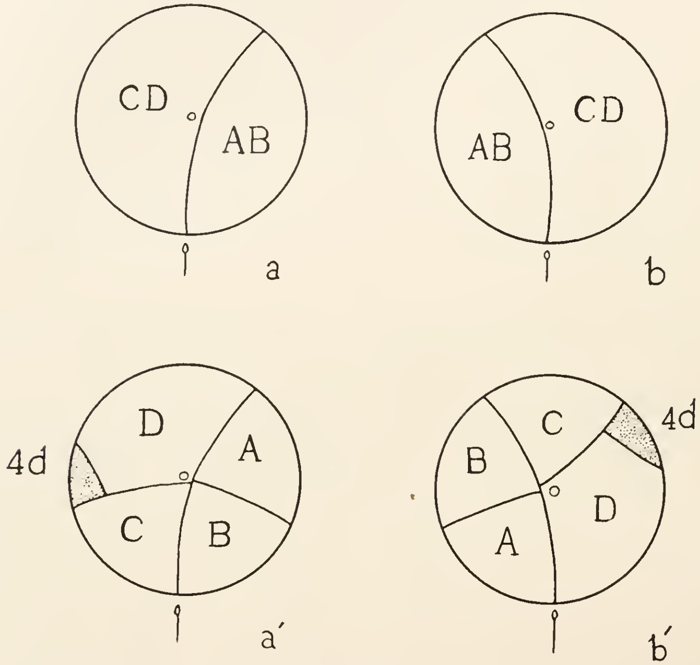

FIG. 7. Diagrams indicating the position of the first cleavage with respect to the polar body, and the entrance point of the spermatozoön; also the location of the 4-*d* cell. In *a*′ the position of the 4-*d* resulting from the type of first division in *a* is shown, in *b*′ that in *b*.

The Cleavage of Nereis

The egg of *Nereis* is particularly well suited for a study of relation of entrance point to cleavage, not only because the slow entrance of the sperm makes for accuracy of observation, but also because after the sperm-head has entered, a portion is left sticking to the fertilization membrane, and, if exactly on the horizon, may be still seen at the time when the cleavage begins. The technique was the same as for the *Cumingia* eggs, but since the egg is larger, thicker supports made from glass tubing were used. Owing to the great thickness of the jelly a relatively large space between the cover and slide is essential. The location of the first cleavage with respect to the entrance point of the sperm has been studied by Just. The observations reported here were made to determine not only the constancy of the relation, but also to determine whether the *AB*-cell always forms to one side of the entrance point—a relation not previously reported. It was found that whereas the *AB* lay to the right in a very large number of cases, there were a few cases where it lay to the left. Nevertheless, at the four-cell stage only one arrangement of blastomeres was found (even in those with *AB* to the left), namely, that shown in Figs. 7, *a'*, or 7, *b'*.

The first plane of cleavage coincided with the entrance point in 33 cases. In 17 cases it was less than 45°. In 14 cases it was more than 45° and less than 90°. It is apparent from these observations that the agreement (51 per cent) is far from perfect.

Of the 33 cases of close coincidence, the first plane passed to the right of the pole in 28 cases, and in five cases to the left. Of the 17 cases less than 45° away, it passed to the right in 11 cases, and to the left in 6 cases. This conclusion was reached by arbitrarily shifting the entrance point to the nearest surface point in the cleavage plane. Here again there were more cases where *AB* lay to the right than to the left.

The configuration of the cells after the second cleavage is always of the same type (Fig. 7, *a'*, or 7, *b'*), whether the first cleavage passes to the left or to the right of the pole. In the 28 cases in which the first plane passed to the right, the *D*-cell formed away from the entrance point and in the five cases in which it passed to the left the *D*-cell formed near the entrance point.

The third cleavage of *Nereis*, as is well known, is always dexiotropic. The succeeding divisions of the egg alternate left and right. Hence, in both sets of cases the 4-*d* cell comes to lie near the second cleavage plane, which Wilson has shown to be near the median plane of the body.

In 1912 Just reported results of experiments on *Nereis* eggs, in which the entrance point was marked by the path of India ink in the jelly. He found coincidence varying from 50 per cent in one set to 60, to 80, to 95 per cent in other sets. He placed emphasis on those sets in which the greatest amount of agreement occurred. The exceptions he supposed were due to errors of technique, since by a change in technique he found in one set of 60 eggs, 100 per cent coincidence. Our own results gave only 51 per cent exact coincidence. That the vaseline we used was not injurious was shown by removing the eggs from the slide after the 4-cell stage and finding that they produced normal trochophores. We tried the India ink method in the hope of obtaining a large number of observations from a single preparation, but abandoned it because of the uncertainty in many cases of following the marker exactly to the surface of the membrane, and unless this can be done with absolute certainty there remains too great a chance of making a wrong inference, especially when the coincidence is not quite exact. In our opinion continuous observations on single eggs, while much more tedious, are safer.

Discussion

The main interest in these observations concerns the two types of the four-cell stages in *Cumingia*. As pointed out, one type arises in eggs in which the *AB*-cell forms to the right of the entrance point, and the other where it forms to the left. Since these two types give rise to two different planes of bilateral symmetry, *on the assumption made,* the problem of the determination of these planes seems to resolve itself into the problem of what determines that the cleavage plane lies to one or to the other side of the pole. Since these two types appear with equal frequency in *Cumingia,* it may seem that it is only a matter of " chance " to which side of the pole it passes. In *Nereis* there is only one type of four-cell stage and the *AB*-cell in the majority of cases (85 per cent) forms to the right of the entrance point. To this extent it conforms to the rule found for *Cumingia.* Since the *AB*-cell of *Nereis* lies to the right of the entrance point in 85 per cent of cases, its location does not here seem to be a matter of chance. In *Chætopterus* there is again only one type of four-cell stage, but here the *AB*-cell lies equally often to the right or to the left of the entrance point. Since there are here three different types of behavior leading to the formation of normal embryos, it may be inadvisable at present to try to reduce them all to one mechanism. The spiral type of cleavage common to all these eggs might incline one to attempt to find an explanation of the fact that the first cleavage plane passes to the right (with respect to entrance point) or to the left consistently in the different types. In *Cumingia* the egg regulates according to whether the *AB*-cell lies to the right or to the left of the entrance point. In *Chætopterus,* although the *AB*-cell again may lie either to the right or left of the pole there is no regulation, because the second cleavage plane coincides with the median plane. In *Nereis* no regulation is necessary, in this sense, in the majority of cases because these all conform to the same rule, but in the few exceptional cases the result is the same as in *Chætopterus.*

As already stated, an examination of the second cleavage spindle of *Cumingia* has not shown a spiral arrangement of the spindles. It is equally obvious, however, that, just prior to the division, the spindle in the *CD*-cell lies well to one side, indicating the future position of the *C*-cell. After the division, the *A* and *C* blastomeres approach each other, more nearly in the polar than in the antipolar hemisphere in both types, while the *B* and *D* cells meet in a straight line at or near the antipole. If this be taken as evidence for a spiral second cleavage, then there are both leiotropic and dexiotropic second cleavages in *Cumingia.* Since the third cleavage is always dexiotropic this would contradict the " law " of alternating spiral cleavages.

It has been pointed out in the text that the two types of cleavage of *Cumingia* give rise to two different planes of bilateral symmetry. In one type the median plane coincides with this first cleavage plane, and in the other type with the second. This conclusion, however, is based on the assumptions that the law of alternating cleavage holds from the third cleavage on, and that the 4-*d* blastomere gives rise to the germ bands.

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THE EFFECT OF LOW OXYGEN TENSION ON THE PULSATIONS OF THE ISOLATED HOLOTHURIAN CLOACA

BRENTON R. LUTZ

(From the Bermuda Biological Station for Research,[1] *the Mount Desert Island Biological Laboratory, and the Physiological Laboratory of Boston University School of Medicine)*

The sequence of events in the respiration of *Stichopus moebii* Semper has been adequately set forth by Crozier (1916). In laboratory aquaria the rhythmic activity of the cloaca is distinctly periodic. A series of several pulsations is followed by a pause during which water is expelled from the respiratory tree. Then another series of inspirations begins. The number of inspirations in a series was found by Crozier (1916) to range from five to eleven, the greatest number being found in the largest animal. Pearse (1908) pointed out that, if the respiratory pulsations of *Thyone briareus* are prevented for some time by repeated mechanical stimulations, the contractions which ensue when stimulation ceases are greatly augmented in amplitude.

Oxygen deficiency has often been associated with periodicity and augmentation of response in various tissues. Douglas and Haldane (1909) have described periodic breathing in man under low oxygen tensions, and Douglas (1910) found the same type of breathing at high altitudes. Magnus (1904) and Frey (1923) reported that a stoppage of the oxygen supply to beating smooth muscle results immediately in an increase in amplitude. The present paper deals with the phenomena which have been observed on decreasing the oxygen available to a rhythmically beating isolated strip of circular muscle from the cloaca of *Stichopus moebii* Semper. This holothurian is found in great numbers in the shore waters at the Bermuda Biological Station. During the summer of 1927 the author repeated some of the experiments on a ring preparation from the cloaca of *Cucumaria frondosa,* very abundant at the Mount Desert Island Biological Laboratory, Maine.

METHOD

Crozier (1916) has shown that the cloaca *in situ* in the isolated posterior end of *Stichopus* will maintain its pulsations for many hours. No reference to the use of an isolated strip of this organ could be found in the literature. The present work was carried out with an opened ring of the circular muscle of the cloaca. A cloacal-end preparation was first made similar to that described by Crozier (1916). The cloaca was then excised by cutting the radial muscles with a scalpel and freeing the organ from the anal rim by a transverse cut. From the muscular tube thus obtained a strip was made, one to two centimeters broad, and from four to six centimeters long. This strip was suspended vertically in a vessel of sea water by means of an L-shaped glass rod and a counterbalanced aluminum lever. A 250 cc. graduated cylinder cut off to hold about 125 cc. was found convenient as a vessel to hold measured amounts of sea water, or through which sea water could be made to flow continuously. The temperature of the water was recorded and found to vary little during an experiment, or from day to day. Therefore no special precautions for maintaining constant temperature were necessary.

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[1] Contribution number 158.

RESULTS

Records were taken from strips of *Stichopus* cloaca beating under the following conditions: (1) in a continuous flow of sea water, (2) in a limited amount of sea water, (3) in boiled sea water with added carbon dioxide, (4) in boiled sea water of various degrees of aeration, and (5) in normal sea water with potassium cyanide added.

Continuous Flow of Sea Water.—When sea water was made to flow continuously through the vessel at the rate of about 100 cc. a minute, the strip beating therein gave a tracing which was exceedingly uniform over a period of several hours, as may be seen in Fig. 1. Both amplitude and tone increased during the first hour. This condition was maintained for an hour or more. Then the tone began to fall very gradually while the amplitude remained about the same. After five to seven hours from the beginning, the amplitude began to decrease slightly. The rhythm was exceedingly regular and no indications of periodicity appeared. The rate of beat decreased slowly from the start, in one case almost 50 per cent after seven hours and forty-one minutes; but the preparation was still vigorous and regular.

Limited Amount of Sea Water.—When a strip was allowed to beat in a limited amount of sea water, that is in 100 cc. without change, the amplitude began to increase in about three hours and distinct periodicity developed as seen in Fig. 2. The increase in amplitude continued for an hour or more, becoming 230 per cent in one case. The tone was maintained until the increase in amplitude occurred, when it gradually fell; but the increase in amplitude was not entirely due to a decrease in tone since the contractions of the strip raised the lever a greater

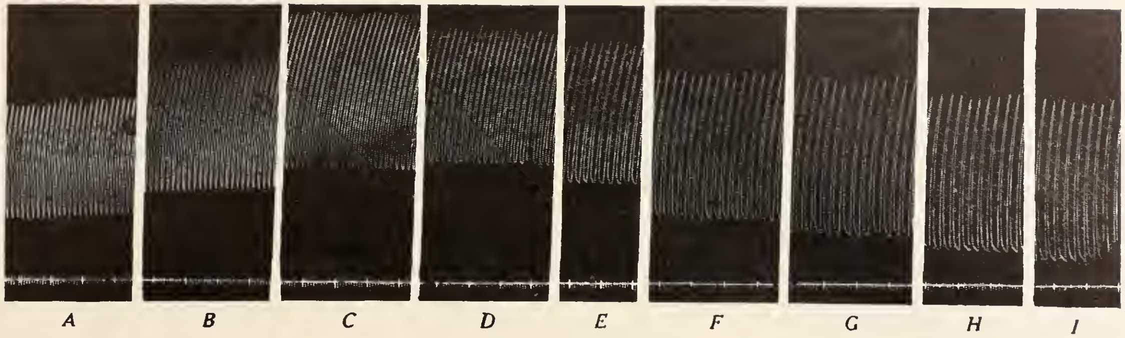

Fig. 1. Experiment 47. An isolated cloacal strip from *Stichopus moebii* beating in sea water flowing continuously through the vessel. In this and subsequent tracings the large divisions on the base line mark time in minutes. Time after immersion of strip: *A*, 12 minutes; *B*, 45 minutes; *C*, 81 minutes; *D*, 139 minutes; *E*, 210 minutes; *F*, 300 minutes; *G*, 345 minutes; *H*, 390 minutes; *I*, 435 minutes. Duration of experiment 7 hours and 45 minutes.

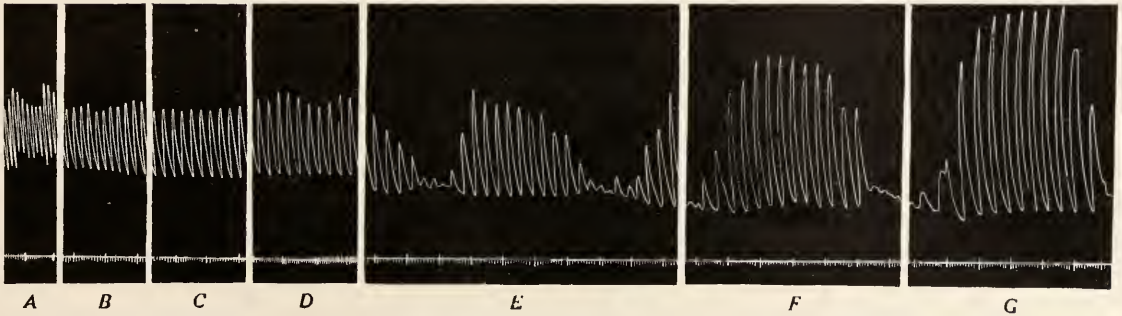

Fig. 2. Experiment 44. Cloacal strip beating in 100 cc. of sea water. Time after immersion of strip: *A*, 1 minute; *B*, 28 minutes; *C*, 60 minutes; *D*, 121 minutes; *E*, 161 minutes; *F*, 185 minutes; *G*, 222 minutes. Duration of experiment 3 hours and 48 minutes.

distance above the base line than in the beginning. Finally both amplitude and tone fell markedly. The rate of beat decreased constantly from five or six at the beginning to two or three per minute during the periods of beating. The length of the periods of inhibition of beat gradually increased to three or four minutes.

Boiled Sea Water.—Sea water which had been boiled in a narrow-necked flask and cooled to laboratory sea water temperature (28° C. or 29° C.) was used. When the muscle strip was immersed in 100 cc. of this water, the first two or three beats usually increased in amplitude, but both tone and amplitude almost immediately fell and the strip ceased beating in from three to five minutes as shown in Fig. 3, *A*. If the water was aerated within three minutes by sucking it into a hypodermic syringe and squirting it back forcibly, a partial recovery occurred, which showed periodicity at first but later an uninterrupted rhythm (see Fig. 3, *C*). Several attempts to bring about recovery after waiting a longer period failed. The pH of the boiled sea water (indicator method) was sometimes as high as 8.8 as compared with 8.1 to 8.3, the pH for unboiled sea water in this region.

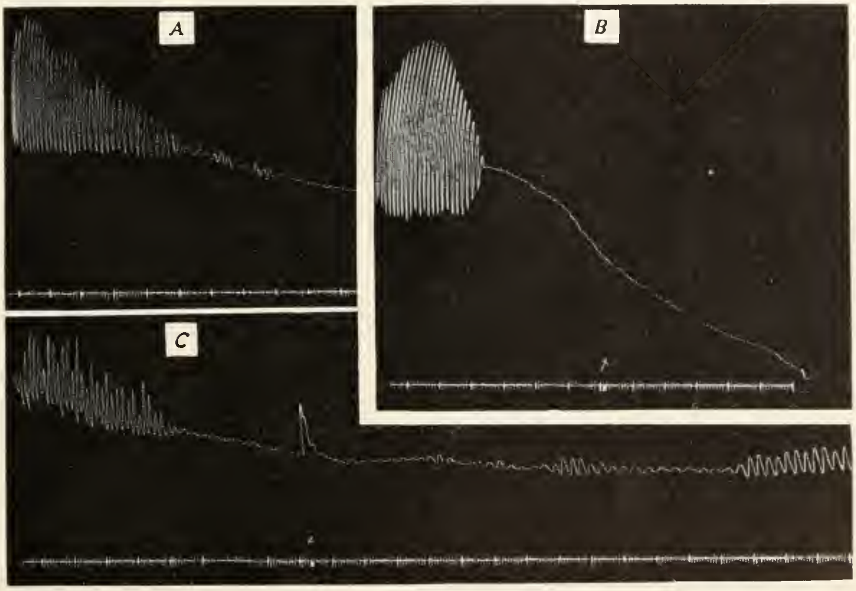

FIG. 3. *A*. Cloacal strip in 100 cc. of boiled sea water, pH 8.4. *B*. Cloacal strip in 100 cc. of boiled sea water treated with carbon dioxide, pH 5.8. Aeration at *X*. *C*. Cloacal strip in 100 cc. of boiled sea water, pH 8.3. Aeration at *X*. pH 8.2 immediately after aeration. pH 8.2 after 102 minutes.

Boiled Sea Water with Added Carbon Dioxide.—Inasmuch as boiling removed the carbon dioxide as well as the oxygen, the former was replaced by means of a carbon dioxide generator. This resulted in boiled sea water ranging from pH 5.8 to 7.7. At the latter value the strip ceased to beat in three and one-half minutes and at the former value cessation occurred in three minutes. Aeration of the water after a three minute period of cessation failed to induce recovery (see Fig. 3, *B*). It seems therefore that neither the lack of carbon dioxide in the boiled sea water nor the increased alkalinity was the cause of the cessation of the pulsations.

A moderate excess of carbon dioxide was produced by treating 125 cc. of unboiled sea water with carbon dioxide until the pH was 7.0. This procedure was brief and probably did not remove much oxygen. In experiment 75 (Fig. 4) the amplitude began to decrease slowly after an hour, the rate decreasing gradually from the beginning. Neither augmentation of amplitude nor periodicity had appeared when the experiment was stopped after two hours and fifty-six minutes. When, however, an excess of carbon dioxide was produced by adding a few drops of N/10 HCl to a preparation beating in 100 cc. of unboiled sea water, there was an immediate rise in tone and increase in amplitude which soon gave way to a fall of tone and amplitude and finally to cessation of beat. It is therefore not probable that an accumulation of carbon dioxide in the immersion fluid as a result of tissue activity in a limited volume of water is the cause of the appearance of periodicity although it might be called upon to account for the increase in amplitude.

Boiled Sea Water of Various Degrees of Aeration.—When a decreased oxygen content of the sea water was produced, either by mixing boiled sea water with unboiled sea water or by partial aeration of boiled sea water, the augmentation and periodicity appeared much sooner than when a limited volume of unaltered sea water was used, the onset varying from a few minutes to two hours, according to the degree of oxygen lack. In one case the boiled sea water had been stored for several hours in a narrow-necked flask with only a few square centimeters of water surface exposed to the air. A strip beating in 100 cc. of this water became periodic at once and each successive period showed an increase in amplitude which finally amounted to about 200 per cent. The tone and the rate of beat, however, fell rapidly.

In another experiment in which 100 cc. of boiled sea water had been partially aerated, wave-like variations in amplitude appeared 13 minutes after immersion, and gradually developed into periodicity 53 minutes after the start. The amplitude increased from 10 mm. to 24

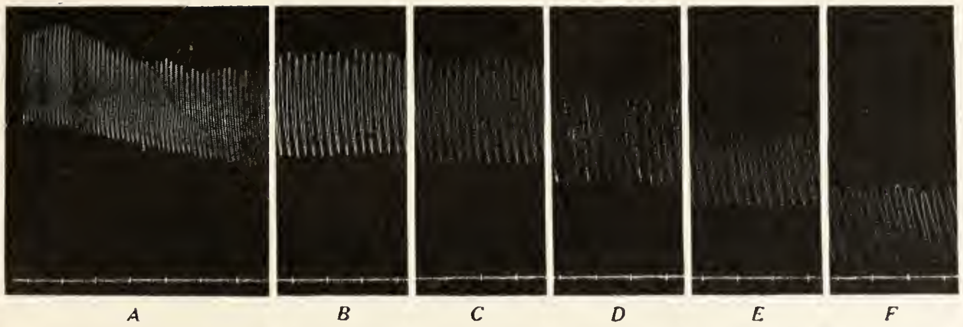

Fig. 4. Experiment 75. Cloacal strip in 125 cc. of sea water treated with carbon dioxide, pH 7.0. *A*, beginning at 10:00; *B*, 10:30; *C*, 11:00; *D*, 11:30; *E*, 12:00; *F*, 12:52. At the end, 12:56, pH 7.0.

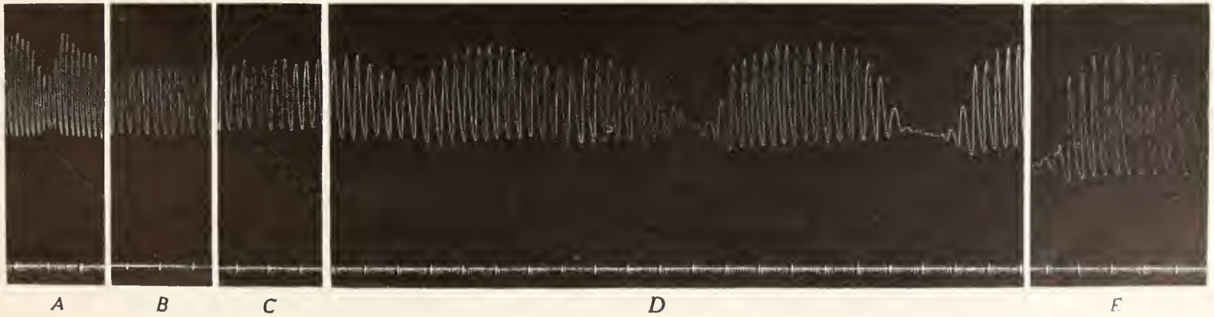

Fig. 5. Experiment 62. Cloacal strip in 100 cc. of boiled and partially aerated sea water. Before boiling, pH 8.3. After boiling and partial aeration, pH 8.4. Time after immersion: *A*, 5 minutes; *B*, 32 minutes; *C*, 97 minutes; *D*, 117 minutes; *E*, 181 minutes. pH 8.4 after 166 minutes.

mm. and was still high when the experiment was stopped at the end of 93 minutes. The tone fell rapidly during the first five minutes and then more slowly during the next ten minutes after which it was unchanged. The rate decreased about fifty per cent during the first half hour and then remained constant.

In experiment 62 (Fig. 5) the boiled sea water (100 cc.) was partially aerated. Before boiling the pH was 8.3, but after boiling and partial aeration it was 8.4. The amplitude of a strip beating in this water decreased at first with a tendency to form waves. Then for a period of 80 minutes the amplitude remained constant, but at the end of this period the amplitude began to increase, becoming 65 per cent greater than that during the previous period of uniform amplitude. Periodicity appeared in about two hours from the beginning of the experiment, the number of beats in each period ranging from ten to sixteen, while the period of interruption varied from one minute and a quarter to three minutes. The pH was still 8.4 about forty-five minutes after periodicity and augmentation were well developed. Apparently these phenomena were not due to increased acidity of the surrounding medium, nor was the cessation of beat in the cases of extreme oxygen lack due to an increase in the concentration of the salts resulting from boiling.

When 75 cc. of boiled sea water were mixed with 25 cc. of unboiled water, the pH of the mixture was 8.8. The first few contractions increased in amplitude about 20 per cent, lasting for about three minutes. Then a fall occurred, and the amplitude remained uniform in height until waves in amplitude appeared in one hour indicating the onset of periodicity, which became well marked about half an hour later. At this time the pH was still 8.8.

Effect of Potassium Cyanide.—When ten drops (about 0.5 cc.) of $M/10$ potassium cyanide were added to 100 cc. of sea water in which a strip had been beating for a few minutes, the results were similar to those obtained with partial aeration. An increase in amplitude occurred within two minutes which varied in different cases from 18 to 400 per cent. The tone increased at about the same time. Periodicity occurred within fifteen minutes. In one case it began in three minutes, and the rate of beat was increased about one beat per minute for a brief period after the addition of potassium cyanide. Finally the tone and amplitude fell and the strip ceased to beat (see Fig. 6).

An examination of the results presented above suggests that lack of oxygen is responsible for the appearance of the two chief phenomena noted. Since augmentation and periodicity did not occur with a continuous flow of water but did occur in three hours when the amount of water was limited to 100 cc., one might expect that one or more of several factors were responsible, such as, an increase in carbon dioxide, an increase in unoxidized acids, a depletion of essential ions, or a depletion of oxygen. However, when the carbon dioxide content of sea water was increased at the beginning, the phenomena did not appear, although a temporary increase in amplitude and tone could be produced upon addition of hydrochloric acid. Moreover, in the experiments in which the phenomena did appear, the pH of the sea water was either unchanged or decreased very slightly. Since both augmentation and periodicity were made to appear much sooner when the water was partly depleted of oxygen at the beginning, or when potassium cyanide was added, the inference is that oxygen lack was either directly or indirectly responsible.

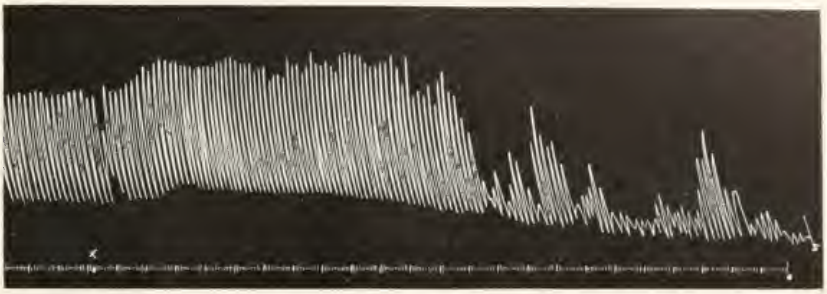

FIG. 6. Experiment 82. Cloacal strip in 100 cc. of sea water. Time of immersion 4:28. At X, 4:35, 20 drops of $M/10$ potassium cyanide added.

When a ring preparation made from the cloaca of *Cucumaria frondosa* was allowed to beat in a limited volume of sea water, namely, 25 or 30 cc., periodicity appeared in 50 minutes on the average in eight out of ten preparations. Two showed no periods. Augmentation of amplitude occurred in five cases. When boiled sea water was used the periodicity appeared in 25 minutes on the average in 14 out of 15 preparations. One showed no periods. Augmentation of amplitude occurred in 13 preparations. These results, especially when considered in the light of the results on *Stichopus,* indicate that lack of oxygen is a factor tending toward an early development of periodicity and augmentation.

DISCUSSION

Periodicity is a part of the normal respiratory sequence of a holothurian, the rhythmical contractions of the cloaca being inhibited while the body muscles squeeze out the sea water from the respiratory tree through the relaxed anal valve. Crozier (1916), however, found no evidence of periodicity in the cloacal-end preparation of *Stichopus* and came to the conclusion that the stimulus for spouting has its origin outside the cloaca. It should be noted that he used larger volumes of water than were used in the work reported in this paper. Apparently no oxygen deficiency existed in his preparations, in which the cloacal pumping probably produced a sufficient movement of water to keep it aerated beyond the needs of the preparation. Since the isolated cloacal strip will exhibit regular periods of inhibition, the inference is that a part of the normal mechanism for spouting lies within the cloacal muscle. Since periodicity is lacking with sufficient aeration and appears quickly under conditions of oxygen deficiency, one is inclined to believe that low oxygen tension is a factor in determining the normal respiratory sequence in the holothurian.

Periodicity is commonly observed in the respiratory activity of vertebrates, as for example in the breathing of hibernating animals, in Cheynes-Stokes respiration, and in respiration at high altitudes. The causes of this phenomenon are usually associated with the chemical conditions in the respiratory center. Most authors have offered explanations which concern directly or indirectly the hydrogen ion concentration of the blood or fluid surrounding the cells. Gesell (1925), however, has called attention to the hydrogen ion concentration within the cells of the former, pointing out that when oxygen is present carbon dioxide is formed, but if oxygen is lacking lactic acid results. In either case the activity of the center increases as the acidity rises. Assuming a critical level, one needs further to call upon a mechanism for altering either the level or the acidity to account for periodic inhibition.

The augmentation of amplitude observed with a decrease in the available oxygen is in accord with the work of Magnus (1904) and of Frey (1923), who worked on vertebrate smooth muscle. Gross and Clark (1923), in an investigation on the influence of the oxygen supply on the response of the isolated intestine to drugs, stated that cutting off the oxygen resulted in a decrease in amplitude and tone. They did not comment on the immediate brief increase in amplitude and tone shown in their published graphs. The literature offers many additional observations which indicate that a certain degree of oxygen lack results in increased activity of tissue. Kaya and Starling (1909) found that lowering the oxygen tension resulted in excitation in the whole nervous system. Sherrington (1910) found that a certain degree of asphyxia favored the elicitation of the scratch reflex, and suggested that the hyperexcitability of the reflex was due to oxygen lack. Mathison (1911) showed by the use of hydrogen, nitrogen, and carbon monoxide that the initial effect of oxygen lack on the medullary centers is clearly stimulating. Gasser and Lovenhart (1914) found by the use of carbon monoxide and sodium cyanide that decreased oxidation stimulated the medullary centers at first but later depressed them. Kellaway (1919) demonstrated that lack of oxygen may lead to stimulation of the adrenal glands, and Lutz and Schneider (1919) have observed a dilatation of the pupil in men during a period of breathing nitrogen. They also presented evidence to show that the cardiac and the respiratory medullary centers in man respond very quickly to changes in the partial pressure of oxygen. A decrease in oxygen tension increased the activity of these centers, while an increase in oxygen tension decreased their activity. Glazer (1929) found that intravenous injection of sodium cyanide in a dog increases the reflex response of the anterior tibial muscle, and Winkler (1929) obtained a similar effect with low alveolar oxygen tension.

In the muscle-and-nerve-net preparation reported in the present paper, it appears that the carbon dioxide content and the acidity of the surrounding fluid are not primary factors in controlling its activity. This conclusion is supported by the work of Hogben (1925) who found that, on adding acid to the perfused heart of *Maia* and of *Homarus,* the pH could be lowered from 7.0 to 5.6 without producing a change in the mechanical phenomena. Reduction beyond this point produced an immediate effect on the character of the rhythm. Nor could any alteration be noticed in the beating of the smooth muscle of *Helix* and of *Aplysia* on changing the pH from 7.0 to 6.0. In fact it is possible that the pH outside of the cell may vary markedly without greatly altering that inside of the cell. The oxygen tension appears to have some influence on tissue acidity. Frey (1923) presented evidence which shows that without oxygen the tissue rather than the surrounding fluid first changes its hydrogen ion concentration, and if this approaches the optimal value, an increased ability to respond ensues. The anaerobic production of acid in cellular activity and the rôle of oxygen in the recovery process suggest that oxygen lack is acting indirectly when cellular activity is first increased and is subsequently depressed.

Summary

1. An isolated muscle strip from the cloaca of *Stichopus moebii* Semper and a ring of muscle from the cloaca of *Cucumaria frondosa* were used in sea water as rhythmically beating preparations.

2. In a continuous flow of sea water the contractions (*Stichopus*) were nearly uniform in rate and amplitude over a period of several hours, but a gradual decrease in both finally occurred.

3. In a limited volume of sea water (100 cc.) the amplitude (*Stichopus*) began to increase after three hours and a distinct periodicity of the regular rhythm developed. In the case of the cloacal ring of *Cucumaria* beating in 25 or 30 cc. of sea water, periodicity appeared in 50 minutes on the average.

4. In boiled sea water the strip (*Stichopus*) ceased beating in from three to five minutes, but partial recovery took place if the water was aerated within three minutes. If the carbon dioxide was replaced in the boiled sea water, cessation of beat occurred as before. In 25 or 30 cc. of boiled sea water the ring of cloacal muscle from *Cucumaria* developed periodicity in 25 minutes on the average.

5. A moderate excess of carbon dioxide in sea water (pH 7.0) did not bring on augmentation nor produce periodicity.

6. In boiled sea water of various degrees of aeration the augmentation of amplitude and the periodicity appeared sooner than in unboiled sea water. When little oxygen was present both phenomena appeared almost immediately, while the pH of the surrounding fluid was unchanged.

7. When potassium cyanide was added to the sea water an increase in amplitude and tone occurred and periodicity appeared.

8. Evidence from the literature is cited supporting the view that decreased oxygen tension results at first in increased activity of muscular and nervous tissues. This view is further supported by the evidence presented in this paper.[2]

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PHENOTYPICAL VARIATION IN BODY AND CELL SIZE OF *DROSOPHILA MELANOGASTER*

W. W. ALPATOV

(*From the Institute for Biological Research, Johns Hopkins University*)

I.

The purpose of this paper is to contribute to the solution of the question of the relationship of the cell size and body size, using well-known and standard material. The literature devoted to this question is very extensive, but most of the work done cannot be considered to fulfil the requirements of exact experimental investigation, in regard either to the control of conditions, or the homogeneity of the material, or the precision and accuracy of the treatment. Comparatively modern compilations of the data available have been made by Levi (1906) and Martini (1924).

Concerning the more limited problem of the correlation of body size and cell size in Diptera there have been two recently published papers. Loewenthal (1923) attacks a problem which corresponds to one part of the present investigation, namely the influence of underfeeding on the body and cell size of the blow-fly. The first criticism which may be made of Loewenthal's work is that he does not give any indication of the ages of the normal and underfed maggots. It therefore is not clear whether the observed smaller size of the hypodermis cells is due to differences in the age of larvae or in the feeding. At the same time Loewenthal does not find any difference in the cell size of the gonad rudiments, in spite of their difference in size. The following conclusion is reached (p. 91): " Danach ist die Körpergrösse der ausgebildeten Imagostadiums unabhängig von der Zellgrösse und allein bedingt von der mehr oder minder grossen Zellanzahl." Further a totally incorrect statement is made concerning the absence of cell divisions during the larval life (p. 92): " Mit Abschluss der Embryonalentwicklung stellen die larvalen Zellen ihre Vermehrungstätigkeit ein, das ganze Wachstum der Larve von wenigen mm Länge nach dem Schlüpfen aus dem Ei bis zur Länge von 2 cm einer verpuppungsreifen Ruhelarve beruht allein —wenn man von den während der Larvalperiode für die Gesamtgrösse nicht ins Gewicht fallenden Imaginalanlagen absieht—auf dem Grössenwachstum der Zellen." Przibram's and Megušar's (1912) investigations showed that this is not the case in the postembryonal development of *Sphodromantis* (Orthoptera, Mantidae) and I (1929) have shown also that the metamorphosis of *Drosophila* is connected with six simultaneous divisions of the cells of the whole body.

The same subject of the relationship of the size of an organ and the size of the cell has been touched upon by Bridges (1921, 1925). In both of his papers differences in the cell structure, namely, nuclear structure, are shown to be connected with the size of the whole body and its organs. It was discovered that these intersex-producing females (triploid) could be identified by their somatic characters, namely, large coarse bristles and large roughish eyes (1921, p. 253). In the second paper it is the size of the ommatidia which is shown to be different in flies having different chromosomal complexes. "The cells of triploid individuals are readily seen to be larger than the cells of diploids, and correspondingly their facets are larger" (Bridges, 1925, p. 709).

I became interested in the problem of body size and cell size years ago while working on the oceanographic expedition of the Floating Marine Scientific Institute to the Russian arctic seas. The first expedition in 1921 gave very impressive material on the geographical variation in the dimensions of the body of different marine animals. It could be particularly easily shown on such a group of animals as Isopoda, which have a postembryonal development ending with a definite imaginal stage analogous to that of insects. Extensive biometrical data on variation of Isopoda, taken from localities with different temperatures, showed perfectly that the colder regions (for instance, the Kara Sea) are populated by races which have a larger body size than regions with warmer water temperature (Barents Sea). On the second expedition I strove to collect some material on the histology of local races of some of the species of Isopoda. But the severe conditions of navigation during this and following summers did not allow the accomplishment of this intention. During the winter of 1927–28, working at this Institute, I succeeded in working out a more or less accurate method of producing *Drosophila* imagoes of different sizes, using two factors, temperature and underfeeding. The method of counting the number of hairs on the wings of *Drosophila* as a method of estimating the number of cells on a certain surface of the wing was discovered by a friend, Dr. Th. Dobzhansky (1929), who was kind enough to explain it to me. I have the pleasure to express also my deepest gratitude to Dr. Raymond Pearl for criticism and valuable suggestions.

II.

Two factors have been used in producing flies of an abnormal size. It was shown in an earlier paper that the first of them was the low temperature, which decreases the rate of development and produces flies of a larger size (see Alpatov and Pearl, 1929). The method of collecting new-born larvæ has already been described (Alpatov, 1929). Flies belonging to Wild Line 107 have been taken for parents of our experimental animals, the collected larvæ being 0–2 hours old at the moment of putting them on food. The bottles had been planted with yeast 2 hours before the putting on of larvæ, and watered with a few drops of distilled water. Electric and low temperature Hearson incubators were used for keeping the bottles with flies. Five bottles with 50 larvæ each were kept at 18° C., five others at 28° C. The development from the moment of the populating of the bottle till the moment of the pupation was more than twice as long in the cold series as in the warm. It is unnecessary to discuss here at length the question of temperature and development rate, this having been done in another paper (Alpatov and Pearl, 1929). The technique of breeding in the experiment with underfeeding was the same except for the fact that the yeast was put in the bottles with synthetic medium at the moment of populating the bottles with larvæ.

A method of getting undernourished larvæ by taking larvæ from the food before the normal end of feeding has been used by various workers, for instance, Ezhikov (1917, 1922), Smirnov (1926, 1927), Cousin (1926), Herms (1928) and others. Most of these authors did not attempt to determine with sufficient accuracy the moment of taking the larvæ from the food, Herms being in that respect an exception. In the present investigation, larvæ were taken from the food exactly 48 hours after the moment of populating the bottles with 0–2 hour-old larvæ. Larvæ which reached the desired age were taken from bottles and placed in half-pint bottles containing plain agar. The mouths of the bottles were covered with 40 mm. watch glasses and sealed with plastaline used in modelling. This was done in order to prevent the larvæ, which become very active, from crawling out. The day after the larvæ had turned into pupæ the watch glasses were replaced by the usual cotton stoppers.

Table I shows that the larvæ with a subnormal period of feeding pupate earlier than normally fed ones. This can be compared with Kopeč's (1924) statement that ". . . if we begin to apply starvation to older specimens during developmental stages . . . the transformation of these animals is accelerated." A little longer prepupal development of the normally fed larvæ, those which served as controls to the underfed being compared with the 28° flies of the early October experiment, cannot be very easily interpreted. It might exist in a difference in conditions—perhaps a difference in yeast growth which lengthened the duration of development of larvæ in the second set of experiments.

TABLE I

Data on the Conditions of the Development of Flies Reared for the Study of the Problem of Cell Size

	Temperature limits of variation	Average	Time of the beginning of the experiment	Time from egg until pupation, in hours	Time of feeding
Underfed flies	Kept at 28°	—	October 24, 1928	80.39±.50	48 hours
Normally fed flies	Kept at 28°	—	October 24, 1928	93.16±.74	Until normal leaving of the food
28° flies	27.1–28.9°	28.2°	October 8, 1928	87.40±.36	Until normal leaving of the food
18° flies	17.0–20.0°	18.2°	October 8, 1928	200.86±.89	Until normal leaving of the food

The flies have been collected in 70 per cent alcohol and measured in glycerine under a cover glass. The following characters on the wings of collected flies have been studied: the length and width of the wing, and the number of hairs on a surface equal to 0.1 square mm. on the lower surface of the wing. Fig. 1 represents the points of measurement and the place where the hairs have been counted.

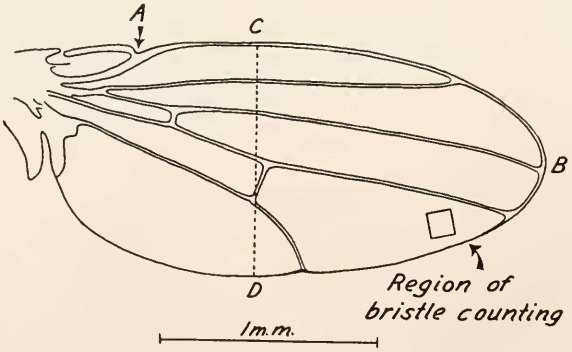

FIG. 1. Measurements of the wing. *AB*, length of the wing, *CD*, width of the wing. The square shows the area of the bristle countings.

For the measurements the following optical systems were chosen: Spencer 25.4 mm. objective and a micrometer ocular in a No. 2 ocular. The countings of the hairs of the lower surface of the wing were done in a way approved by Th. Dobzhansky. Pieces of paper with squares representing 0.1 square mm. at a given magnification have been prepared by projecting through an Abbé camera lucida 0.1 mm. from an object micrometer placed on the microscope stage. A Spencer microscope was used with objective 4 mm. and ocular × 10. The hairs have been projected by means of the camera lucida and drawn with a sharp pencil. Only hairs whose bases happened to fall inside the square have been counted (Fig. 2).

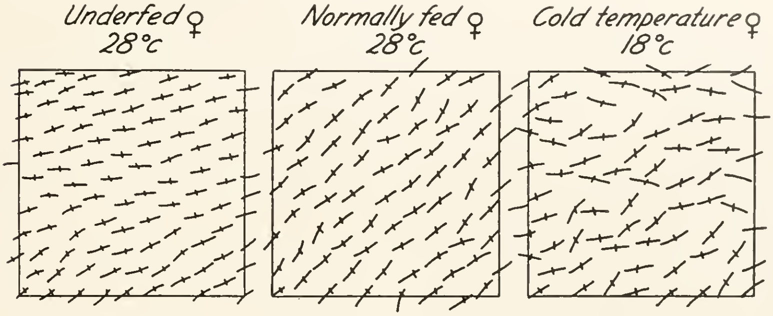

FIG. 2. This figure represents the bristles on the surface of 0.1 mm.^{2} in the lower surface of the wings of underfed, normally fed, and cold temperature females. The bristles which have a line across their middle have been counted, those without lines had their basis outside the limits of the 0.1 mm.^{2} and have not been counted.

We did not consider it wise to count the hairs exactly at a certain point (in so many parts of a millimeter from a certain vein) as has been done by Dobzhansky. There are two reasons for not doing so. First of all the distribution of hair on that part of the wing is more or less uniform. On the other hand, the wings of underfed and normal are so different in size that a distance expressed in absolute measurement would show morphologically quite different regions. Fifty specimens of each set of underfed, normal fed and 18° flies were studied in regard to the density of the hairs. Dr. Th. Dobzhansky succeeded in finding that on the wings each hair corresponds to a separate cell. This can be seen on specimens of flies just emerged from the pupæ. The wings look opaque and the cells can be distinctly seen. It is very likely that the tiny hair covering the thorax of *Drosophila* corresponds also to hypodermis cells, and their density may also be used as a method of studying the size of the hypodermal cells.

III.

It is desirable at this stage to digress briefly to consider a matter which arose as an extension of the original problem. It is the question of functional relation between the time of larval feeding and the final size of the flies. First of all I reinvestigated the data published by Herms (1928) and found that when plotted on a diagram they reveal a very interesting picture.

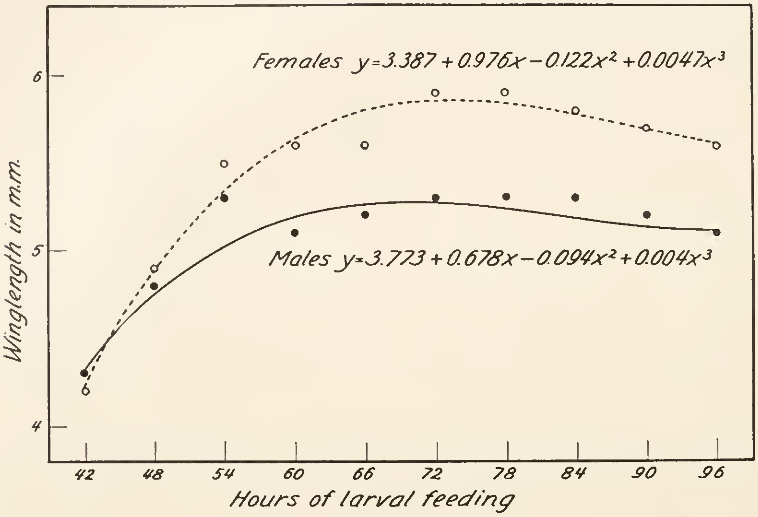

FIG. 3. The relation between the wing length and the length of the feeding period in *Lucilia sericata*. Data from Herms (1928).

Fig. 3 represents Herms' data and two cubic parabolas which I fitted to the observed points. Up to the 78 hour point the trend of the curves represents the upper part of a typical growth curve. There cannot be any doubt that this trend corresponds exactly to the upper branch of the logistic curve which can be fitted to the growth of *Drosophila* larvæ of the third instar (see Alpatov, 1929). But the decline after 78 hours is quite remarkable. Going back to my paper on larval growth in *Drosophila* I was able to find in Fig. 13 particularly a slight indication as to an analogous decline of the size of the larvæ killed at the latter end of the life of the culture. It was therefore decided to clear up this question on specially collected material. This was done in April 1929. Forty bottles containing 0.500 grams of Magic yeast with 25 drops distilled water were populated by 80 larvæ each. Five drops of water were added every day during the larval growth. The experiment was run at a temperature of 25° C.

Table II contains data on the sex relations in the material studied. Let us first compare the percentage of males emerged from larvæ taken from the food at 48–80 hours, which is equal to 102.6, with that of males emerged from larvæ taken from food at the age of 84–96 hours, in which case the percentage is 89.9. This difference finds its explanation in the fact that male larvæ in our case started pupation earlier than females, which is shown by the very high percentage of males among larvæ pupated naturally at the age of 84–96 hours—187.2 per cent. On the whole the group of bottles which was taken to get larvæ fed 84–96 hours shows a percentage of males equal to 109.1. Comparing it with the sex proportion in normal undisturbed bottles where we had

TABLE II

Absolute and Relative Numbers of Larvæ, Pupæ and Adult Flies in the Experiment on Underfeeding of Larvæ

Hours from the beginning of feeding	Number of larvæ taken from the food	Number of the pupæ observed	Number of pupæ unable to produce flies	Number of flies emerged				
				Total	In per cent of the larvæ	Male	Female	Male in per cent of female
48	151	—	17	30	19	18	12	150.0
52	170	—	7	80	47.1	44	36	122.2
56	152	—	11	87	57.2	37	50	74.0
60	158	—	12	123	77.8	62	61	101.6
64	162	—	2	154	95.1	72	82	87.8
68	170	—	7	153	90.0	85	68	125.0
72	151	—	12	117	77.6	69	48	143.8
76	135	—	3	127	94.1	56	71	78.9
80	150	—	1	142	94.7	70	72	97.2
Total 48–80	—	—	—	—	—	513	500	102.6
84	86	36	2	78	90.7	39	39	100.0
88	137	12	4	115	83.9	62	53	117.0
90	111	30	11	100	90.1	56	44	127.3
92	110	46	3	100	90.1	45	55	81.8
94	95	61	10	79	83.2	30	49	61.2
96	85	73	6	75	88.2	27	48	56.3
Total 84–96	—	—	—	—	—	259	288	89.9
Flies emerged from pupæ at 84–96 hours								
84	—	—	—	—	—	25	11	227.3
88	—	—	—	—	—	6	5	120.0
90	—	—	—	—	—	23	8	287.5
92	—	—	—	—	—	30	12	250.0
94	—	—	—	—	—	32	23	139.1
96	—	—	—	—	—	45	27	166.1
Total 84–96	—	—	—	—	—	161	86	187.2

356 males and 415 females, we find that the normal percentage of males is 85.8. We can therefore draw the conclusion that there is a definite preponderance of males among flies emerged from the underfed larvæ. In other words it seems that a selective process makes the male more resistant to underfeeding.

TABLE III

Wing Length, Width and Relative Width of the Flies Emerged from Larvæ taken from the Food at Different Hours

Hours	Males				Females			
	Length	Width	Index	Number	Length	Width	Index	Number
48	1.107	.6490	58.6	17	1.207	.6972	57.8	12
52	1.164	.6847	58.8	25	1.239	.7154	57.7	25
56	1.331	.7847	59.0	25	1.413	.8034	56.9	25
60	1.321	.7697	58.3	25	1.493	.8459	56.7	25
64	1.394	.8145	58.4	25	1.572	.8898	56.6	25
68	1.409	.8289	58.8	25	1.588	.9102	57.3	25
72	1.406	.8428	59.9	25	1.561	.9083	58.2	25
76	1.371	.7983	58.2	25	1.511	.8493	56.2	25
80	1.412	.8261	58.5	25	1.586	.8938	56.4	25
84	1.476	.8833	59.8	25	1.673	.9349	55.9	25
88	1.440	.8516	59.1	25	1.641	.9321	56.8	25
90	1.472	.8777	59.6	25	1.646	.9255	56.2	25
92	1.423	.8468	59.5	25	1.614	.9032	56.0	25
94	1.426	.8457	59.3	25	1.613	.9077	56.3	25
96	1.444	.8686	60.2	25	1.608	.9083	56.4	25

TABLE IV

Wing Length, Width and Relative Width of the Flies Emerged from Pupæ Pupated at a Given Hour, and of Those Emerged from Pupæ Pupated during the Whole Pupation Period

Hour	Males				Females			
	Length	Width	Index	Number	Length	Width	Index	Number
84	1.484	.8805	59.3	24	1.709	.9901	57.9	11
88	1.490	.8887	59.6	6	1.728	.9944	57.5	4
90	1.493	.8876	59.5	15	1.715	1.007	56.7	8
92	1.456	.8516	58.5	25	1.649	.9312	56.5	12
94	1.455	.8499	58.4	25	1.644	.9389	57.1	23
96	1.463	.8544	58.4	25	1.672	.9536	57.0	25
Normal pupation	1.475	.8745	59.3	40	1.673	.9668	57.8	40

Tables III and IV give the average length and width of wings of our material. The wing length is graphically represented in Fig. 4.

With the exception of some cases (72, 76 and 80 hours) the material confirms what could be seen on curves based on Herms' data. The most interesting thing is the declining slope of the curves toward the end. It is not only with underfed flies that this decline is noticeable, but the flies normally pupated in the beginning of the pupation period had longer wings (*i.e.*, larger bodies) than flies in which pupation has been delayed.

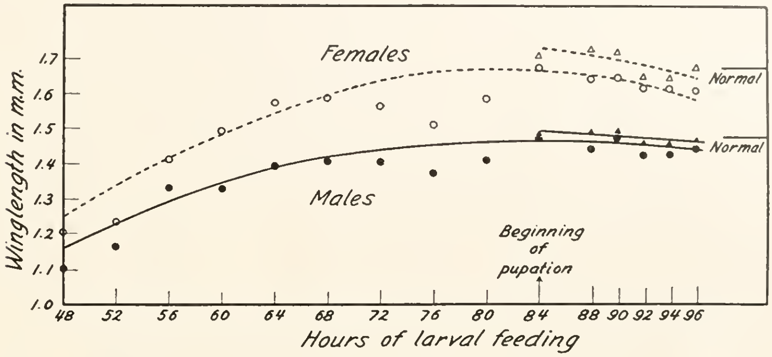

FIG. 4. This figure represents the relation of the length of the wing and the length of the larval feeding in *Drosophila melanogaster*. The triangles indicate the length of wings of flies pupated at certain hours.

Table V gives the statistical proof of this conclusion. It can be definitely seen that in males and females without regard to whether the pupation is going naturally or the flies emerge from larvæ taken from the food, those which pupate first are larger than those which pupate later. We may express the observed phenomenon in a little different form. There is a negative correlation between the duration of larval life and the size reached during growth. The faster the larva grows the sooner it reaches the pupal stage. We take the liberty of comparing our case with the experiments on *Cucumis melo* described by Pearl in his book, *The Rate of Living,* (1928). The larvæ which reach a larger size in a short time have naturally a higher rate of growth than larvæ which remain small for a longer time. Therefore the statement brought forward by Pearl (p. 139) that "between growth rate and duration of life to the beginning of death the correlation is negative and significant in degree" can be perfectly well applied to our case.

We do not know whether these differences arise really as a result of inherent vitality or are the result of differences of treatment of larvæ during the population of the bottle. Further experiments have to solve this question. Our results are very close to Kopeč's discovery (1924) of the negative correlation between the duration of larval period

TABLE V

Length of the Wings (in mm.) of Flies Emerged from Larvæ Taken at a Given Hour from the Food, from Pupæ Formed at a Certain Hour, and from Control Bottles

Males

			Difference and ratio			
Larvæ taken from the food at 84–88 and 90 hours. $N = 75$	Mean	1.458±.004	0.27±.005	Larvæ taken from the food at 92–94–96 hours. $N = 74$	Mean	1.431±.003
	Standard deviation	.0450	$R=5.4$		Standard deviation	.0405
	C. of V.	3.08±.17			C. of V.	2.83±.16
Difference and ratio	—	.025±.005 $R=5.0$	—	Difference and ratio	—	.026±.004 $R=6.5$
Pupated at 84–88–90 hours. $N = 45$	Mean	1.433±.003	.022±.004	Pupated at 92–94–96 hours. $N = 74$	Mean	1.457±.002
	Standard deviation	.0265	$R = 5.5$		Standard deviation	.0277
	C. of V.	1.79±.13			C. of V.	1.90±.11
—	—	—	—	Pupated during the whole pupation period. $N = 40$	Mean	1.469±.004
					Standard deviation.	.0393
					C. of V.	2.68±.20

TABLE V—*Continued*

Females

			Difference and ratio			
Larvæ taken from the food at 84–88–90 hours. N = —	Mean	1.650±.004	.043±.005	Larvæ taken from the food at 92–94–96 hours. N = 75	Mean	1.607±.003
	Standard deviation	.0545			Standard deviation	.0432
	C. of V.	3.30±.18			C. of V.	2.69±.15
Difference and ratio	—	.064±.006 $R=10.7$	—	Difference and ratio	—	.047±.004 $R=11.75$
Pupated at 84–88–90 hours. N = —	Mean	1.714±.004	.060±.005	Pupated at 92–94–96 hours. N = —	Mean	1.654±.003
	Standard deviation	.0312			Standard deviation	.0339
	C. of V.	1.82±.18			C. of V.	2.05±.13
—	—	—	—	Pupated during the whole pupation period. N = —	Mean	1.672±.005
					Standard deviation	.0507
					C. of V.	3.03±.23

and the weight of the chrysalids in *Lymantria dispar* (L.). This negative correlation found in twelve experimental groups out of sixteen is particularly well expressed in males.

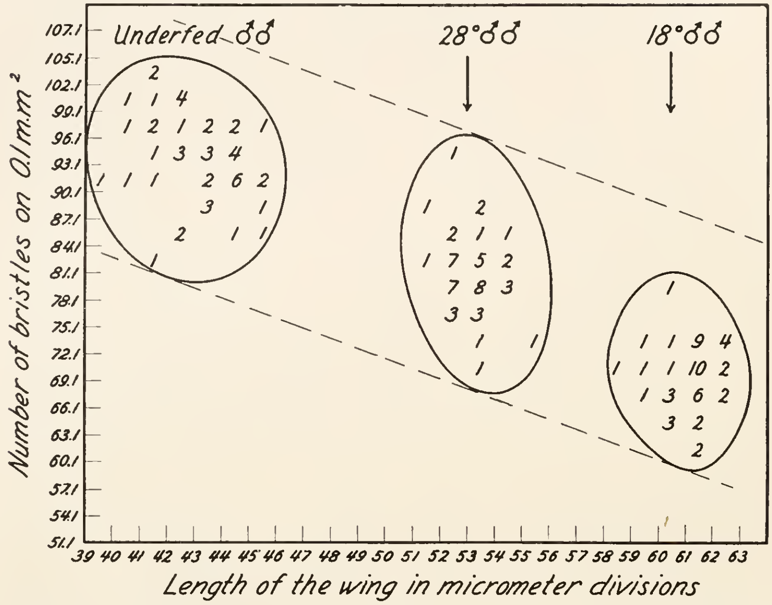

FIG. 5. Correlation between the length of the wings and the number of bristles per 0.1 mm.² on the lower surface of the wings of the male.

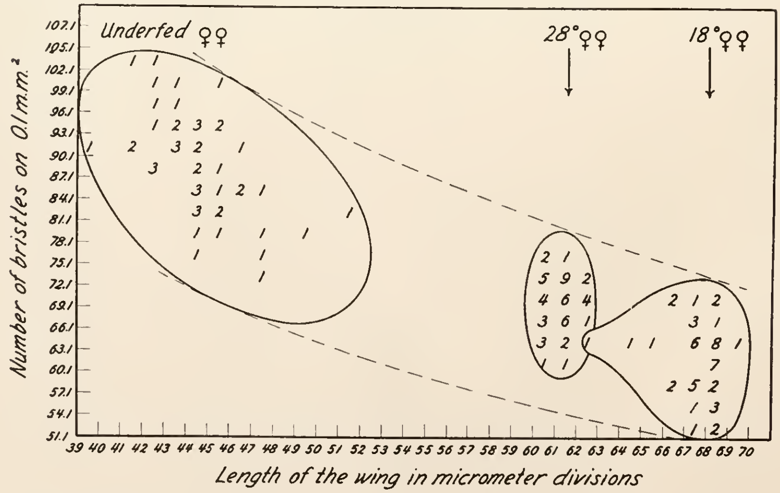

FIG. 6. Correlation between the length of the wings and the number of the bristles per 0.1 mm.² on the lower surface of the wings of the females.

TABLE VI

Biometrical Constants for the Length of the Wings and the Number of the Bristles per 0.1 mm.^{2} of the Lower Wing Surface, as Well as the Coefficient of Correlation between These Characteristics

	Underfed flies		Normally fed flies		28° flies		18° flies	
	Number of the bristles per 0.1 mm.^{2}	Length of the wing in mm.	Number of the bristles per 0.1 mm.^{2}	Length of the wing in mm.	Number of the bristles per 0.1 mm.^{2}	Length of the wing in mm.	Number of the bristles per 0.1 mm.^{2}	Length of the wing in mm.
Males								
Mean	94.00±.46	1.224±.004	81.04±.39	1.507±.002	—	1.488±.002	70.00±.34	1.737±.002
Standard deviation	4.836	.0456	4.125	.0230	—	.0227	3.600	.0240
C. of V.	5.14±.35	3.73±.25	5.09±.34	1.53±.10	—	1.53±.10	5.14±.35	1.38±.09
r	−0.299±.086 $R = -3.5$		−0.024±.094				+0.098±.093	
Females								
Mean	89.14±.68	1.283±.006	70.36±.35	1.737±.002	—	1.716±.002	63.50±.46	1.917±.005
Standard deviation	7.089	.0590	3.691	.0190	—	.0215	4.773	.0498
C. of V.	7.95±.54	4.59±.31	5.25±.35	1.09±.07	—	1.25±.08	7.52±.51	2.60±.18
r	−0.563±.064 $R = -8.8$		+0.075±.094				+0.101±.093	

IV.

Correlation tables shown in Figs. 5 and 6 contain the basic data on the number of hairs on 0.1 mm.^{2} and length of the wings. The horizontal axis gives the wing length in divisions of the ocular micrometer, each division being equal to 28.333 microns. Table VI represents constants derived from Figs. 5 and 6 with the addition of wing length of 28° flies. The wing length is expressed in millimeters.

TABLE VII

Average Width of the Wings and Width Index, i.e., Width Expressed in Per Cent of the Length

	Underfed flies		Normally fed flies		28° flies		18° flies	
	Width of the wing	Index	Width of the wing	Index	Width of the wing	Index	Width of the wing	Index
Males . . .	.7151	58.23	.8970	59.33	.8871 ± .0021	59.62 ± .11	1.024	58.85
Females .	.7253	57.29	1.015	58.30	1.004 ± .002	58.51 ± .12	1.102	57.31

Let us discuss the influence of the factor under consideration on the wing as a whole. Table VII gives us the constants for the width in millimeters as well as the width in percentage of the length. There is a pronounced sex difference in the size of the wing, the females in all groups being larger than the males. The relative width of the wing is larger in the males, as can be seen by comparing males and females in all groups, and particularly those of the 28° group. The difference is 6.9 times larger than its probable error. (The indices in this case have been calculated by the use of Pearson's formula.) Another point of interest concerning the relative width of the wing is that in the females as well as in the males the underfed and 18° flies seem to have narrower wings than the " normal " 28° flies. The sex difference is also influenced by abnormal conditions. Table VIII shows that in " normal " 28° conditions the sex difference is the greatest, while underfeeding and low temperature reduce the difference. The lower line in Table VIII contains recalculated data from the experiment described in a former paper (Alpatov and Pearl, 1929). The effect of low temperature and consequently of the slow development can be seen in this case also. It is difficult to find an adequate explanation of this phenomenon, which very likely is connected with certain differences in male and female postembryonal development, that is, with different time of the manifestations of different characters during the larval or pupal life.

Turning our attention to the main problem of our investigation, one glance at the correlation tables shows that the larger the size of the wing of the corresponding group of flies, the smaller the number of cells on the area of 0.1 mm.[2] In other words, the larger flies, considering *inter-group* variation, have also larger cells. The coefficients of correlation for each of the six groups of flies have been calculated separately. They are given in Table VI. Only in the case of underfed males and females is the correlation significant and negative. The conclusion is that in underfed flies the size of the body is negatively correlated with the number of cells on a definite surface of the wing. A possible but very dubious explanation of the absence of such correlation in the case of normally fed and cold temperature flies might be that the variation in the wing length of *Drosophila* developed from normally fed larvæ is so small that the correlation could not manifest itself.

TABLE VIII

Sex-Index of the Wing Length, i.e., Male Wing Length Expressed in Per Cent of the Female

When studied	Underfed flies	Normally fed flies (28°)	28° flies	18° flies
1928	95.33±.54	86.76±.15	86.71±.15	90.61±.26
1927	—	—	88.18±.16	91.93±.18

So far as the variation of the flies belonging to different groups is concerned, it can be seen that the coefficient of variation of the number of cells does not show any definite difference in different groups. At the same time the variation of underfed flies in the length of the wing is much greater than that of the flies which had a normal feeding, no matter at what temperature. Previous investigators who have worked on variation of flies under conditions of under-feeding have also described the increasing variation of experimental animals (see Smirnov and Zhelochovtsev, 1926).

We have now to approach the problem of the actual surface-size of the cells and its relationship to the size of the whole organ. Table IX represents all the calculations relating to this question. The surface of a cell in square microns was determined by dividing 10,000 microns (0.1 mm.[2]) by the number of hairs on that surface. It can easily be seen that the larger the flies the greater the surface of the cell. Another point of interest is the pronounced sex difference in the size of the cells, the females having much larger cells than the males. This has been pointed out by Dobzhansky (1929). The next step was to

TABLE IX

Biometrical Constants and Indices for Different Characteristics of the Experimental and Control Flies. The last three columns show these characteristics expressed in percentages of the values for the 18° flies.

Males

Character	Underfed flies	28° flies	18° flies	Underfed flies	28° flies	18° flies
Surface of a cell	106.32±.52	123.40±.59	142.86±.69			
Square root of the surface = "length" of a cell	10.31±.07	11.09±.08	11.95±.08	86.3	92.8	100
Calculated length of a cell	8.42±.08	10.37±.08	11.95±.08	70.5	86.8	100
Length of the wing	1.224±.004	1.507±.002	1.737±.002	70.5	86.8	100
Females						
Surface of a cell	112.18±.86	142.13±.71	157.48±1.14			
Square root of the surface = "length" of a cell	10.59±.09	11.92±.08	12.55±.11	88.8	95.0	100
Calculated length of a cell	8.40±.10	11.37±.08	12.55±.11	66.9	90.6	100
Length of the wing	1.283±.006	1.737±.002	1.917±.005	66.9	90.6	100

come from the surface values to linear values which has been done by calculating the length of the cell, which was obtained by taking a square root of the surface of the cell. The data on wing length gave the possibility to calculate the percental decrease in the wing length taking the wing length of 18° flies as a basis. Multiplying by this percental decrease in wing length the number giving the "length" of cell in cold temperature (18°) flies, we obtained the figures represented in our table under the heading "Calculated length of cells." Comparing them with the dimensions obtained by taking the square root, we can easily see that the assumption that the wing length varies proportionally to the length of its constituents does not hold true. The three columns on the right of Table VII represent the changes in wing size and cell size expressed in per cent of 18° (cold) flies. The same relationship between these two characteristics is shown in a percental scale on Fig. 7, the diagonal line represents the relationship in case of a proportional change in wing length and cell length; the dotted line shows the actual percental decrease in cell size in different groups of our flies.

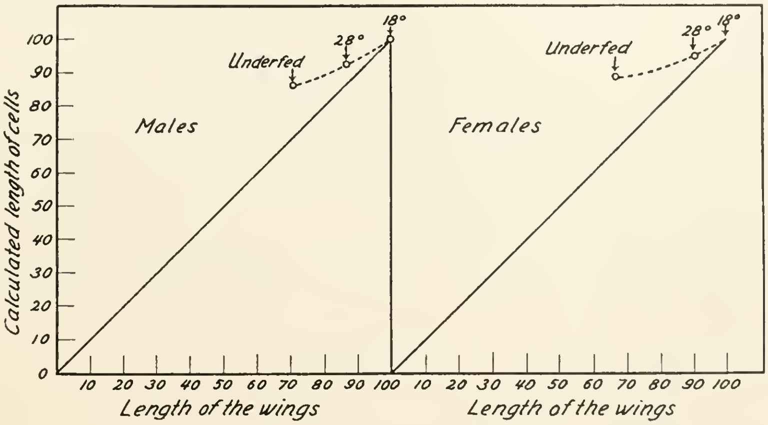

FIG. 7. The dotted lines represent the relationship between the percentage of decrease of the wing length and the percental decrease in the corresponding percental length of the cells calculated by taking the square root of the surface of the cells.

The general conclusion of all these calculations is that the reduced size of cells alone cannot explain the reduction of the organ. The only possible way to explain it is the assumption that the decrease in the organ size—in our case in wing size—is not the result of a decreased size of its cells alone, but also of a reduced number of cells. This last conclusion has a certain bearing upon the problem of the cell constancy in the organism.

If our discussion is correct, the organism can evidently react to the factor decreasing in size not only by decreasing the size of the cells but also the number of cell divisions. The present limited material does not warrant further discussion, but it may be hoped that other investigations in the field of cell-biometry may create a similar basis for understanding the variation of the whole organism as *Die Zelluläre Pathologie* of Virchow did for the interpretation of the pathology of the whole organism.

SUMMARY

1. Dobzhansky's method to determine the number of cells under the surface of the wing membrane of *Drosophila melanogaster* by counting the number of hairs has been used in the present investigation of the relationship of the organ size to the size of its cells.

2. Underfeeding and development at low temperature have been the factors to produce flies under and above the normal size.

3. The functional relation between the time of feeding of larvæ and the size of the wings of larvæ being the expression of the upper part of the logistic larval growth of the third larval instar can be expressed by a cubic parabola.

4. There is a definite tendency for large larvæ (*i.e.,* fast-growing ones) to pupate earlier, which finds a certain analogy with Pearl's correlation that " between growth rate and duration of life (in this case, duration of larval life) to the beginning of death the correlation is negative and significant in degree."

5. As far as all three groups of flies (underfed, normal and cold flies) are concerned the size of the wings is negatively correlated with the number of hairs on a definite surface of the wing when the groups are considered *as wholes* (inter-group correlation). The existence of such a negative correlation could be shown also *within* the group of underfed females and males, but not within the other groups.

6. Expressing in per cent the increase in size of the whole organ and the increase of the linear dimensions of the cells there is a discrepancy in the rate of changes. This leads to the conclusion that the changes in size of the wing cannot be accounted solely by the changes in the size of the cells. The number of cells must play also a certain rôle in this process.

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OSMOTIC PROPERTIES OF THE ERYTHROCYTE

I. Introduction. A Simple Method for Studying the Rate of Hemolysis

M. H. JACOBS

(From the Department of Physiology, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

I.

There is almost no other single type of animal cell which has been so extensively employed in experimental work in the fields of osmotic phenomena and of cell permeability as the mammalian erythrocyte. From the time of the early studies of Hamburger (1886) down to the present day it has been recognized as possessing a number of peculiar advantages as experimental material. Thus, it can be obtained at all times and places in what for practical purposes are unlimited quantities; indeed, the investigator himself carries about with him wherever he goes a never-failing supply of absolutely fresh and normal erythrocytes, ready for use at a moment's notice. Because of the remarkable constancy of its natural environment—the mammalian body—the erythrocyte, unlike certain other cells frequently used for similar studies, may be expected to show only relatively slight variations in its physiological properties from day to day and from season to season. Furthermore, its simple structure and low rate of metabolism prevent complications which are frequently troublesome with other types of material. Removed from the body it can be kept, if not in an unaltered, at least in a usable condition for a longer time than almost any other kind of animal cell. Finally, there are available for its study methods of great simplicity which are not only quantitative but which are also statistical to an extent perhaps nowhere else realized with physiological material.

Because of these striking, and to a considerable extent unique, advantages the erythrocyte would appear to be an almost ideal type of material for studies in which a high degree of quantitative accuracy is desired. A survey of the literature, however, reveals all too frequently a disappointing failure on the part of investigators to obtain results of this character. Not only is there a very common lack of agreement between the conclusions reached by different workers, but even the same investigator is not infrequently forced to acknowledge an inability on repeating his experiments to obtain consistent and reproducible data.

The erythrocyte, in spite of its apparent simplicity, behaves, in fact, as if it were either naturally a highly variable and capricious type of material, or—what is more likely—as if it were peculiarly sensitive to certain environmental factors which with other types of cells are much less troublesome.

In the course of work which has occupied the author for several years and which will be reported in detail in the series of papers of which the present one is the first, the general conclusion has been reached that the erythrocyte is indeed a highly suitable form of material for many types of experimental work and that accurately reproducible results may be obtained with it, but that such results are possible only with a more careful attention to details than is needed with most other forms of physiological material. As a matter of fact, the very simplicity of the mammalian erythrocyte, which in its mature condition is perhaps only questionably to be called a living cell at all, prevents the maintenance by it in a changing environment of the relative internal constancy which is so characteristic of more complicated cells and of entire organisms. The simplicity of the erythocyte is, therefore, rather paradoxically, actually a source of complexity for the experimenter. Furthermore, there are certain special reasons, closely connected with the functions which the erythrocyte has to perform, why its osmotic properties, in particular, are of necessity far more profoundly affected by slight environmental changes than are those of perhaps any other known type of cell. These reasons will be discussed in the second paper of this series.

In general, the relation which the erythrocyte, considered as experimental material, appears to bear to other types of cells is much the same as that which a canoe bears to boats of more stable design. Both the erythrocyte and the canoe when properly handled have very definite and characteristic advantages, but both have the tendency to penalize any carelessness in their management in a prompt and unmistakable manner. Perhaps at some future day this peculiarity of the erythrocyte may be considered rather as an advantage than a disadvantage.

II.

Before considering certain of the peculiarities of the erythrocyte itself it seems advisable to deal with some of the methods which have been employed in the past in studying the osmotic properties of this type of cell, and, in particular, with the one which has been gradually developed by the author and has been used in the experimental work upon which all of the papers of the present series are based. By giving a single description of the method at this point, unnecessary repetitions may later be avoided.

Osmotic changes in the erythrocyte are, in general, always associated with volume changes. This is true whether the changes are of the simple sort produced by the passage of water alone between the cell and its surroundings or of the more interesting and complicated type, so useful in studies of cell permeability, where the movement of water depends upon osmotic inequalities set up by the passage of dissolved substances across the cell boundary. Any quantitative study of osmotic phenomena will therefore involve the measurement of the amount of volume change which occurs in a given experiment, or the rate of this change, or both.

In the case of the erythrocyte there are available two remarkably simple methods for studying volume changes. The first is the hematokrit method introduced by Hedin (1891). By means of it the total volume of all of the cells in a sample of a given suspension is measured, the cells being tightly packed together in a fine graduated tube by centrifugal force. The advantages of this method are, first, its simplicity and, second, its statistical nature, by which the variability of the millions of individual cells is averaged out. Its greatest disadvantage—and this, unfortunately, is a fatal one in many cases—is that the time required to pack the cells into a mass free from intercellular fluid is so great, even with the most powerful centrifugalization available, that the method can be used only to obtain final end points or, at most, to follow volume changes of extreme slowness. For this reason, in the present series of studies, it has been possible to use it only rarely.

A second method, of even greater simplicity, is that of hemolysis. This method, first systematically employed by Hamburger (1886), depends on the fact that when an erythrocyte in swelling reaches a certain volume, which varies not only with the species of animal but also probably with the individual erythrocyte, it loses a sufficient part of its hemoglobin to become invisible, or almost so, both as viewed singly under the microscope or in the aggregate in a suspension in a test tube. In some cases it is possible by appropriate treatment to restore the invisible corpuscles to visibility; in other cases it is not.

The term hemolysis is sometimes applied to the mere disappearance of erythrocytes; at other times it is used to describe their more complete destruction. This double use of the term, while unfortunate, is perhaps unavoidable at present and every author should therefore designate the sense in which he employs it. It will here be used, for convenience, to apply to what for practical purposes is the easier and more certain end point to observe, namely, the disappearance of the erythrocyte from visibility rather than its more or less complete destruction, concerning which there is usually much greater uncertainty. This usage is further justified by the fact that in "osmotic hemolysis" complete destruction is apparently very difficult to obtain. Thus, Adair, Barcroft, and Bock (1921) were unable with water alone to separate the hemoglobin from the cells containing it sufficiently to obliterate certain effects believed to be due to the cells themselves, though this could be done after the addition of ether, which presumably completed the destruction of the cells.

The hemolysis method for studying the swelling of erythrocytes and, indirectly, therefore, the penetration of dissolved substances, possesses the advantage of extreme simplicity. With no apparatus other than a test tube, very fair ideas as to many problems of cell-permeability may be obtained. The apparatus here to be described refines the method to an extent which permits the experimenter to secure results of a really high degree of accuracy. An even greater advantage of the hemolysis method, however, is that it is available for the study of *rates* of swelling, even in experiments of very short duration. In the present series of papers no experiments of a total duration of less than one second will be reported, but the author has pointed out elsewhere (1927) that a principle used with conspicuous success for another purpose by Hartridge and Roughton (1923) can be adapted to the study of hemolytic processes whose duration is only a fraction of a second as is the case, for example, with the hemolysis of the erythrocytes of the sheep in distilled water. In its adaptability to problems involving rapid rates of swelling, and consequently some of the most interesting problems of cell physiology, the hemolysis method is, in fact, of unique importance.

On the other hand, the method possesses at least two disadvantages which must be frankly admitted and then dealt with as adequately as circumstances permit. The first is that hemolysis may be caused or influenced by various factors other than osmotic ones. The disappearance of an erythrocyte does not necessarily indicate that it has by swelling reached some definite hemolytic volume, V_h, though this is frequently the case. It is important, therefore, that certain control experiments shall always be performed before inferences concerning the rate of swelling are drawn from observations on the rate of hemolysis.

These control experiments may take various forms. Thus, in cases where osmotic factors alone are involved, it should be possible to show: (1) that the substance or substances present in the solution in which hemolysis occurs have no observable hemolytic effect when added in varying amounts, up to and preferably exceeding those employed in the experiments, to an isotonic solution of NaCl or some similar non-penetrating substance; (2) that the process of hemolysis by a pure solution of the substance in question may be stopped at will at any desired point by the addition in osmotically suitable amounts of NaCl, saccharose, etc.; or (3) that if a solution of NaCl be chosen which is sufficiently hypotonic to cause the hemolysis of some but not all of the erythrocytes in a given sample of blood, the addition of the substance to the partially hemolyzed suspension causes no increase in the degree of hemolysis. The last mentioned test is a very delicate one, though it is somewhat difficult to employ for reasons to be discussed in the following paper of this series.

A second disadvantage of the method is that even in cases where it is reasonably certain that the occurrence of hemolysis is due to the attainment of a definite volume, V_h, this volume represents merely one point on the swelling curve. As compared with the egg of Arbacia (Lillie, R. S., 1916), (McCutcheon, M., and Lucké, B., 1926) whose volume changes can be measured continuously, the erythrocyte appears capable at best of supplying to the investigator only very meagre information about the course of the swelling process.

This disadvantage, however, is not so serious as it might at first sight appear to be. There is reason to believe that the course of the swelling of the erythrocyte can be represented by a fairly simple equation (Jacobs, M. H., 1928) which permits the entire curve to be calculated approximately when one point on it is known. This question will be dealt with more fully in a later paper. Furthermore, in perhaps most experiments, what is desired is not so much the entire curve of swelling as some general measure of the velocity of the swelling process under various experimental conditions, and this may frequently be obtained by a comparison of the times required under the conditions in question to reach the *same* state of swelling in each case. For work of this type the critical hemolytic volume, V_h, when such a volume exists, is a very satisfactory and convenient criterion for comparison.

One important additional point connected with the use of the hemolysis method remains to be mentioned. Both this and the hematokrit methods are statistical in the sense that millions of cells are employed with each. But whereas the latter measures the total volume of all of the cells together without separating them into groups, the former is complicated by the fact that different individual cells hemolyze with different degrees of readiness, and in determining the time of hemolysis, the cells must, in effect, be divided into groups for separate time-measurements. The size of these groups will depend upon the delicacy of the method employed. When a distinction can be made between, for example, 75 per cent and 76 per cent apparent hemolysis, as is the case with the method about to be described, then the time of hemolysis for the group of cells lying between these limits and consisting of one per cent of the total number may be taken as approximately the arithmetical mean of the times at which the above-mentioned degrees of hemolysis are attained. With a cruder method, or in the region of five or ten per cent hemolysis, where measurements are much more difficult to make, the groups dealt with are of necessity larger and a mere averaging of two times gives correspondingly less accurate results.

Because of the heterogeneous nature of any collection of erythrocytes, it is impossible to speak simply of the " time of hemolysis " for a given sample of blood. Different times must be measured for different groups of cells, or, if desired, a single group may be arbitrarily selected for a given experiment by determining in advance for what particular degree of hemolysis the time shall be measured. In any case, the problem is a much more complicated one than if the blood contained only erythrocytes of uniform physiological properties.

On the other hand, a certain degree of heterogeneity may in some respects be an advantage. Assuming that the different degrees of osmotic resistance of the various cells are dependent chiefly on different individual values of the critical hemolytic volume, V_h, which is a plausible, though as yet an entirely unproved assumption, a possible means is suggested for obtaining more information about the course of the entire swelling curve than could be furnished by a perfectly homogeneous group of cells. The details of such a method still remain to be worked out.

A much more definite advantage of the heterogeneity of a given population of erythrocytes is the following. It is frequently necessary to find a solution of " critical concentration " for a group of cells, *i.e.*, which is just at the point of being able to hemolyze these cells without actually doing so. Cells in such a solution are extremely sensitive test objects for studying the effects of such factors as pH, temperature, etc., as will be pointed out in greater detail in a later paper. If the cells in such a group possessed identical properties, it would require many trials to find the appropriate concentration to the desired degree of accuracy (i.e., to less than 0.001M). With as heterogeneous a group, however, as the erythrocytes in ordinary blood, any concentration within fairly wide limits may be selected with the certainty that there will be present in the blood a group of cells which will exactly " fit " the concentration so chosen. In later papers frequent applications of this principle will be mentioned.

III.

A method suitable for the study by the hemolysis method of the osmotic properties of the erythrocyte should possess the following characteristics. It should allow the degree of hemolysis to be estimated more accurately and more rapidly than the usual laborious and not very exact methods of making cell counts or of making hemoglobin determinations after a preliminary centrifugalization. It should permit the time required for the attainment of a given percentage of hemolysis to be measured accurately, even when the total duration of the experiment is only a few seconds. The usual methods are entirely useless in such cases, and this is perhaps the reason why little work has as yet been done on the rates of any except very slow types of hemolysis. The method should, in the third place, provide not merely for the measurement of the time required to reach some single percentage of hemolysis but for that required for the attainment of many different percentages; otherwise, the heterogeneous nature of a population of erythrocytes may give rise to a type of difficulty that will be discussed in a later paper. Finally, though less essential than the characteristics already mentioned, simplicity of the apparatus itself and convenience in its use would be highly desirable features.

The method here described possesses all of these characteristics. It permits successive determinations of the relative concentrations of cells in different suspensions, as well as of apparent percentages of hemolysis, to be made in a few seconds each, which under favorable conditions are reproducible to one or two per cent. It may be used for the study of all rates of hemolysis where the time measured is more than one second. Furthermore, it permits the measurement not merely of the time required to reach some arbitrarily selected degree of hemolysis but also of the times corresponding to all percentages from zero to upwards of 90 per cent. These measurements, which are extremely easy to make, take the form of permanent kymograph tracings where mistakes in instrumental readings or in the recording of them by the observer are impossible, and where all of the details of the experiment are presented in a way that facilitates ready interpretation. Finally, the apparatus is very simple and inexpensive. A crude but satisfactory form of it can be constructed in an hour out of materials available in any laboratory, and its operation can be mastered in a few minutes. The variety of uses to which it can be put and the degree of accuracy which can be secured with it will be made more evident in the later papers of this series.

In principle, the method is not new. It involves merely the measurement of the turbidity of a suspension of erythrocytes by determining the maximum depth of the suspension through which the image of the glowing filament of a carbon lamp is visible. It is to be noted that what is observed is a distinct image rather than the total amount of transmitted light, as is the case, for example, with the methods of Ponder (1923, 1927) or with the nephelometer. Methods similar to the present one for the study of suspensions have been used or suggested by Vlès (1921), Holker (1921) and others, but they lack certain of its most useful features.

The source of the image is the filament of an old-fashioned carbon lamp. The brightness of the filament is kept constant by the use of a milliammeter to measure and a sliding rheostat to regulate the current flowing through it. For the particular lamp employed, a current of 200 milliamperes has proved to be a suitable one and has been everywhere used except where otherwise specified. If desired, the depth of the suspension may be kept constant and the current measured which under the given conditions makes the filament visible. This method, however, is inferior to the one adopted in being less sensitive and in involving more difficult calibrations.

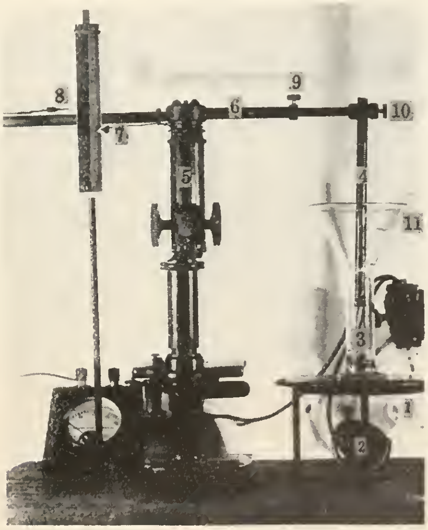

FIG. 1. A simple form of the apparatus, described in detail in the text.

A form of the apparatus somewhat simpler than the one actually employed, but which shows more clearly in a photograph its most essential parts is illustrated in Fig. 1. The image of the filament, 1, is reflected upward by the mirror, 2, through the vessel, 3, in which the suspension to be examined is placed. This vessel is in the form of a tube 2.5 cm. in diameter with a funnel-like expansion above and closed below by a glass plate cemented to the tube with deKhotinsky cement. In cases where it is not necessary to keep the whole apparatus in a water bath for temperature control a separate glass funnel may be substituted.

Into the vessel, 3, plunges a tube, 4, coated internally with a dead-black varnish and closed at its lower end by a small coverglass cemented to it with deKhotinsky cement. It is best always to cover with paraffin any such cement which can come in contact with the solutions used in the experiments. The position of the plunger, 4, is adjusted by means of the rack and pinion of an ordinary microscope, 5, to the tube of which it is attached by the arm, 6. Attached to the microscope are also the pointer, 7, which gives readings on a millimeter scale and the writing point, 8, which touches the smoked paper of a kymograph (not shown). Any movement of 4 is therefore recorded by the kymograph, while at the same time its exact setting can be read from the scale. At the beginning of an experiment the apparatus is adjusted so that a scale reading of zero corresponds to close contact between the bottom of 3 and that of 4. The necessary adjustments of 4 are facilitated by the screws, 9 and 10. Where greater simplicity is desired, a satisfactory substitute for the arm, 6, can be improvised from several ordinary metal clamps.

Since in osmotic experiments on the erythrocyte (as will be pointed out elsewhere) accurate temperature control is essential, the vessel, 3, is usually immersed almost to the top of the funnel in a covered water-bath (not shown in Fig. 1) with blackened interior to cut off all light except that passing through a glass window in its bottom. For the design of this water-bath and for several other features of the apparatus the author is indebted to his assistants, Mr. Arthur K. Parpart and Mr. Wilbur A. Smith.

When the apparatus has been set for a series of experiments it is desirable not to disturb it in changing solutions. This is easily avoided by emptying the vessel, 3, through a removable glass tube (not shown) attached to a filter pump. Another fine-pointed glass tube, also not shown, is usually allowed to dip into the solution in 3. This tube is connected with the compressed air supply and provides in short experiments for rapid and uniform mixing of the blood and the solutions introduced into 3, while in longer ones the current of air may be used as desired to prevent any settling of erythrocytes on the bottom of the vessel. Other tubes connected with the compressed air supply and also not shown provide for the stirring of the water in the water-bath and the prevention of condensation of moisture on the window in its bottom when it is employed at low temperatures.

In using the instrument in an ordinary hemolysis experiment, the procedure is as follows. The tube, 4, is elevated until it is considerably above the position of the expected initial reading. The desired quantity of blood (usually one carefully formed drop from a special pipette) is placed on a small removable paraffin-coated shelf, 11, which is suspended from the side of the funnel. The kymograph is started and the compressed air turned on. Then, as the solution is suddenly poured upon the blood with one hand, the tube is lowered by the other until the image of the filament just appears. The beginning of the experiment is therefore shown by a sudden drop in the line made by the writing point. When the image is seen to increase slightly in brightness, the tube is quickly raised a few millimeters, causing it to disappear. When it again appears the tube is again raised, and this process is repeated until the tube emerges from the liquid. With the apparatus employed by the author and with 25 c.c. of liquid, which is a convenient quantity, this occurs at a scale reading of approximately 60 mm. representing, for samples of blood, in the proportions used, between 80 and 90 per cent apparent hemolysis.

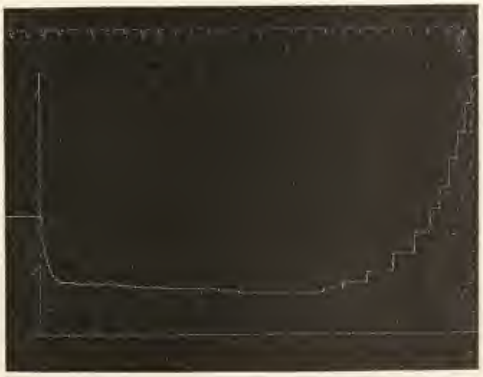

FIG. 2. Typical record of the course of hemolysis of ox blood in 2M ethylene glycol. The time intervals are 5 seconds with every twelfth signal omitted.

The type of record obtained in an experiment of this sort is illustrated in Fig. 2. This particular record gives 11 points on a curve representing hemolysis of ox blood in 2M ethylene glycol. The time intervals marked on the record are of five seconds each with every twelfth one omitted. The slow fall of the curve prior to the sudden rise which indicates hemolysis is due to the gradual recovery by the erythrocytes, with the penetration of the solute, of their initial volumes, and their subsequent further swelling, after a pronounced shrinking has been produced by the concentrated solution employed. Even with solutions of penetrating substances isosmotic with blood, the swelling that precedes hemolysis is usually indicated by a slight fall in the curve. By using greater dilutions of blood so that the readings appear higher on the scale these effects can be considerably magnified and used to good advantage in studying volume changes rather than hemolysis. As would be expected, swollen corpuscles produce lower and shrunken ones higher readings than normal ones, a fact already noted by Holker (1921).

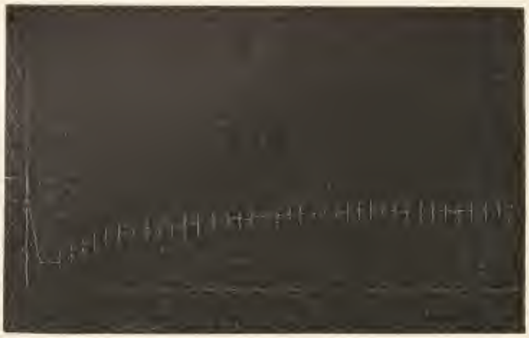

FIG. 3. Typical record of the partial hemolysis of ox blood in 0.082M NaCl. The marks on the curve indicate 30 second intervals.

In experiments of longer duration, where the method described is wasteful of kymograph paper and fatiguing to the eye of the observer, it is preferable to make readings at regular intervals marked by the writing point itself, allowing the drum to move only enough each time to record the level of the reading. A record of this sort covering 18.5 minutes with readings every 30 seconds is reproduced in Fig. 3. It represents the partial hemolysis of ox corpuscles in 0.082M NaCl slightly buffered for pH 7.4 with phosphates.

When the duration of the experiments is very short, *i.e.,* less than perhaps 10 seconds, kymograph records become difficult to make. Fairly complete and accurate hemolysis curves may be obtained, however, in such cases by setting the instrument in advance at any selected point and determining with a stop-watch the time required to reach this point. The vessel is then emptied and the experiment repeated with a different setting of the instrument, and so on, as many times as desired. The complete curve may then be plotted from the separate points obtained.

With experiments of such extremely short duration (*i.e.* less than perhaps 1.5 seconds) that the time required for the uniform mixing of the blood and the solution becomes significant, it is scarcely profitable to attempt to obtain times corresponding to the lower scale readings. Fair accuracy, however, may be secured with sufficiently high settings so that most of the suspension is under the bottom of the inner tube, in which case imperfect mixing is much less serious than otherwise. It is for this reason, as well as because of the fact that the accuracy of the instrument is greater for the higher scale readings, that the author has chosen 75 per cent apparent hemolysis of an approximately 1 : 500 suspension as a very convenient criterion for comparison when for any reason it is necessary to select some single degree of hemolysis for this purpose. With the apparatus used and with most samples of blood this point usually corresponds to a scale reading in the vicinity of 40 mm.

In the use of the instrument several precautions may be mentioned. The only subjective feature of the method is the decision by the observer as to when the filament may be said to be visible. This decision is made with different degrees of readiness and constancy by different persons. The author finds it most convenient so to place the lamp and the mirror that what is seen in the tube is a single small loop of the filament. A reading is taken when the exact form of the entire loop is visible. To secure the greatest sensitiveness of the eye, readings should always be approached from the side of the invisibility rather than from that of the visibility of the filament. In any case, it is important to work fairly rapidly. The image should be approached without hesitation and the reading made without an attempt by moving the tube up and down unnecessarily to secure exactly the right degree of distinctness. What might otherwise be gained in this way is more than lost by the changes that are caused in the sensitiveness of the eye of the observer.

In general, the experience of each individual will soon teach him under what conditions he can secure the most reproducible results. Fortunately, the method permits no possible bias to enter into the measurements, since the observer is unable while making a reading to see the record on the drum, which reproduces with strict fidelity the results of his judgment. It is therefore a very simple matter for anyone using the method to obtain in this way, on a drum moved for the purpose by hand, a series of readings at different levels, which, when subsequently measured, will give exact information as to the reliability of his readings. The readings of the author, in a test of this sort, rarely show a variation of more than 0.3 mm. for a scale reading of 10 mm. or of more than 1.5 mm. for a scale reading of 50 mm. For a suspension whose initial reading is 10 mm. these variations correspond to differences in the estimated percentages of hemolysis of approximately three and less than one per cent, respectively. By averaging a number of readings for a single point, such errors can be still further reduced. The method is therefore seen to be capable of yielding results of a high degree of accuracy.

IV.

The question of the relation between the observed scale readings and the corresponding degrees of hemolysis may now be discussed. Changes in the opacity of the suspension are due primarily to changes in the number of cells which it contains and secondarily to changes in the properties of the individual cells and of the surrounding medium. Since the first mentioned factor is by far the most important, it may be considered first by itself as uncomplicated by, for example, the state

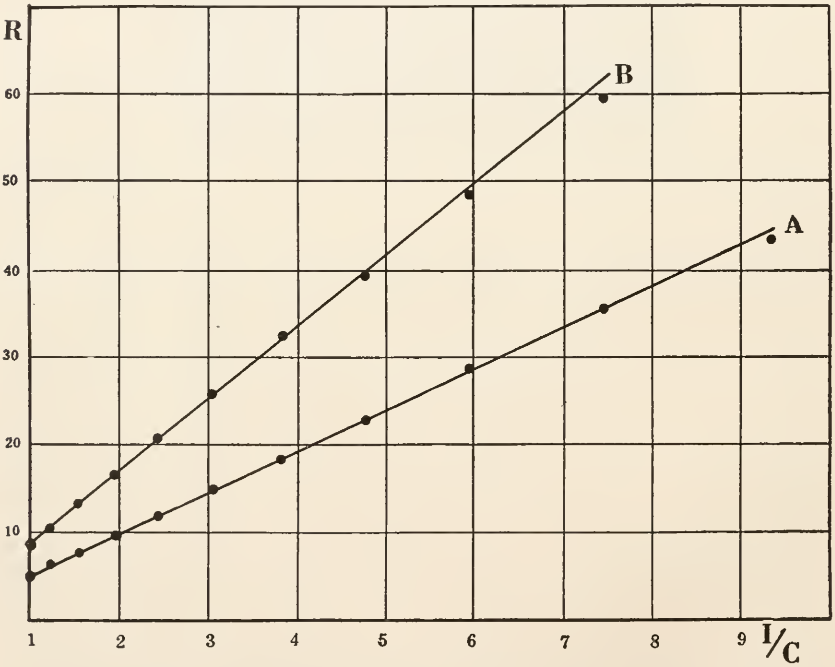

Fig. 4. Effect of dilution of blood on scale reading. Curve *A* represents dilution with 0.9 per cent NaCl of a suspension of ox erythrocytes. Curve *B* represents dilution of a similar but originally less concentrated suspension (approximately 1:500) with a solution containing hemoglobin in the proper amount to give standards representing different degrees of apparent hemolysis; R = scale reading in millimeters and $1/C$ = reciprocal of concentration in arbitrary units.

of swelling of the cells or the presence of hemoglobin in the surrounding solution.

The relation between the concentration of cells in a given suspension and the scale reading of the instrument may readily be obtained by a simple calibration experiment in which a geometrical series of dilutions of an original suspension is used for purposes of standardization. For example, beginning with 125 c.c. of a fairly concentrated suspension of cells in 0.9 per cent NaCl, 25 c.c. are removed for the first measurement and are replaced by 25 c.c. of the salt solution. After thorough mixing this process is then repeated for any desired number of times, a series of suspensions each four-fifths as concentrated as the one preceding it being obtained. Frequently, a factor of dilution of three-fourths or even one-half will give results which are entirely satisfactory with correspondingly less labor.

If now the scale readings so obtained are plotted against the reciprocals of the concentrations, as has been done in the graph labelled *A* in Fig. 4, it will be seen that the points lie almost on a straight line, indicating that the relation between the scale reading R, and the concentration C may be represented approximately by the rectangular hyperbola,

$$CR = \text{a constant.}$$

Actual calculations show that the errors introduced by estimating the relative number of cells in a given suspension, as compared with a standard, by means of this simple relation are usually insignificant. Thus, Table I, from which the data used in constructing graph *A* of Fig. 4 were obtained, shows in columns 1 and 4, respectively, the relative concentrations of cells as determined by actual dilution and as calculated from the relation,

$$CR = R_0,$$

R_0 being the scale reading for the original suspension whose concentration is taken as unity.

It will be observed that the differences amount in no case to more than one per cent, though in other similar experiments differences of two per cent, or rarely more, have been obtained. In general, the differences are greater for low scale readings where the errors of observation are relatively large. Because of the important effect of slight errors in determining the initial scale reading, R_o, an average value for the constant in the equation may, if desired, be obtained from all of the CR products. For comparison with the figures already mentioned there are given in column 5 concentration values calculated in this way. On the whole, they are seen to agree very closely with the values in columns 1 and 4.

As to the simple mathematical relationship found to exist between the number of cells and the observed scale-reading, it may be stated that much the same relation has been reported by Vlès (1921) and Holker (1921) who used methods somewhat similar in principle to the present one, though differing from it in a number of respects, for measuring the opacity of various cell suspensions.

TABLE I

Relation between Scale Readings and Concentration of Erythrocytes in Suspensions

Concentration in arbitrary units = C	Scale readings in millimeters = R (each figure is the average of 10 readings)	Product CR*	Concentration calculated from initial reading $R_0 = 5.0$	Concentration calculated from average of CR products = 4.85
1.00	5.0	5.0	—	—
.80	6.3	5.0	.79	.77
.64	7.7	4.9	.65	.63
.51	9.6	4.9	.52	.51
.41	12.0	4.9	.42	.40
.33	15.0	5.0	.33	.32
.26	18.3	4.8	.27	.27
.21	22.6	4.7	.22	.21
.17	28.6	4.8	.17	.17
.13	35.3	4.7	.14	.14
.11	43.6	4.7	.11	.11

* This product was calculated from more accurate values of C than those in column 1, which are rounded off to two places of decimals only.

The exactness with which relative numbers of cells can be estimated from scale readings, either by calculation or by the use of appropriate standards, particularly for readings above 20 mm., suggests the possibility of using the apparatus, though it was designed primarily for studies of hemolysis, for making the ordinary red-cell counts so frequently needed in physiological and in medical work and for which the laborious and not very accurate hemocytometer method is commonly employed. Preliminary experiments in this direction have shown that by first diluting the blood so that the resulting suspension gives a reading on the more sensitive part of the scale, successive independent determinations differing from one another by no more than one or two per cent may be obtained at will. The time required for each determination, exclusive of that required for cleaning and drying the blood pipette is approximately 15 seconds. With the enormously more laborious hemocytometer method, successive counts, as is well known, usually vary by at least five per cent. Of course, the method gives only relative and not absolute numbers of cells (though it can be made absolute within the limits of the hemocytometer method itself by means of one preliminary cell count) and the readings obtained with it are affected by any variation in the size and shape of the erythrocytes in different samples of blood, as well as by their numbers. The errors to be expected from these sources, however, under the usual physiological conditions are not likely to be as great as those constantly and unavoidably associated with the far more difficult method now almost universally employed.

In using the instrument to estimate percentage of hemolysis, several factors in addition to the concentration of cells must be considered. In the first place, during hemolysis not only do the cells decrease in number, but the hemoglobin liberated from them and contained in the surrounding solution absorbs light and therefore tends to produce lower scale readings than correspond to the mere number of cells. This complication may be dealt with readily, however, by making the dilutions in the calibration series with a solution containing the concentration of hemoglobin that would result from complete hemolysis of the cells. Such a solution is readily prepared by adding to distilled water twice the quantity of blood contained in the same volume of the standard suspension and then, after complete hemolysis has occurred and the solution is entirely transparent, mixing with it an equal volume of sodium chloride solution of twice the concentration of that desired.

As a matter of fact, it turns out that with the dilution of blood that is otherwise most convenient to work with (approximately one part of blood to five hundred of solution) the effect of the hemoglobin on the reading of the instrument, while detectable, is, practically, almost negligible. Under these circumstances the product:

$$(100 - \text{per cent hemolysis}) \times \text{scale reading}$$

proves to be almost constant, as is indicated in graph B of Fig. 4 where the scale readings plotted against the reciprocals of the percentage of unhemolyzed cells lie almost on a straight line.

The theoretical apparent percentages of hemolysis represented in the prepared standards in this particular experiment and the corresponding figures as calculated by the equation

$$\text{per cent hemolysis} = 100\left(1 - \frac{R_0}{R}\right)$$

are given in Table II in columns 1, 3 and 4, respectively, and are seen to be in better agreement than might, from the nature of the case, reasonably have been expected.

For many purposes, therefore, with a very fair degree of accuracy, apparent percentages of hemolysis may simply be calculated from initial scale readings as if the presence of hemoglobin in the external solution could be disregarded. For such calculations, a graphic method, which perhaps requires no explanation here, has been found to save much time. In cases where higher concentrations of erythrocytes are employed or where special accuracy is required, however, appropriate standards for calibration should be prepared.

TABLE II

Relation between Scale Readings and Apparent Percentages of Hemolysis

Apparent percentage of hemolysis represented by standard	Scale reading (each figure is the average of 5 readings)	Percentage hemolysis calculated from initial scale reading	Percentage hemolysis calculated from average of CR products
0	8.6	—	—
20	10.4	17	20
36	13.0	34	36
49	16.6	48	50
59	20.6	58	60
67	25.7	67	68
74	32.2	73	74
79	39.2	78	79
83	48.5	82	83
87	59.6	86	86

The assumption has so far tacitly been made that a given per cent of hemolysis may be represented by a mixture of unaltered cells and of completely hemolyzed cells. This is, unfortunately, not strictly true. In the first place, any solution which is sufficiently dilute to cause osmotic hemolysis of any of the cells must of necessity cause swelling of all of the unhemolyzed cells. In the second place, the possibility must be considered that cells which have not as yet undergone hemolysis may have, nevertheless, given up some of their hemoglobin to the surrounding solution. Both of these factors might be expected to have optical effects which would considerably complicate the situation as so far outlined.

With regard to the first factor, an approximate allowance may readily be made for it by taking as the initial reading for purposes of calculation, not that for a given suspension in 0.9 per cent NaCl, but that for a similar suspension in a solution which is decidedly hypotonic though not quite sufficiently so to cause any hemolysis. This concentration may readily be determined by experiment; for ox blood it is usually in the vicinity of $M/8$ NaCl. Figures obtained in this way by calculation or by calibration with standards made up as before but with the use of hypotonic instead of isotonic solutions, are undoubtedly more accurate than those secured with the neglect of this precaution. It is impossible, however, because of the heterogeneous nature of the material dealt with, to prepare by a simple method of mixtures standards which reproduce with complete fidelity the conditions in a partly hemolyzed sample of the blood.

Even more troublesome is the second difficulty mentioned above. If osmotic hemolysis is, as is maintained by Saslow (1929) an " all or none " phenomenon, then the preparation of standards representing fairly well a given degree of hemolysis is perhaps possible. If, on the other hand, as is believed by Báron (1928), this is not the case, but in a given mixture of hemolyzing cells some have undergone complete hemolysis (in the sense of becoming completely invisible), while others have lost lesser amounts of hemoglobin which can be expected to vary greatly with the conditions of the experiment, then not only is it impossible to prepare standards representing accurately different percentages of hemolysis, but the term percentage of hemolysis itself ceases to have any very exact meaning.

Under these circumstances, and until there is more general agreement than there is at present as to whether osmotic hemolysis is or is not an " all or none " phenomenon, it is perhaps unprofitable to try to introduce into our methods refinements which may have little real significance. It seems preferable merely to speak, as has already been done, of an apparent or an approximate percentage of hemolysis, using for our estimations some convenient though arbitrary type of standard. Figures of this sort will have a considerable value, if used with a recognition of their limitations. In any case, regardless of the type of standard employed, such figures will usually involve an uncertainty of only a few per cent in the assumed degree of hemolysis.

In the absence of any general agreement at present as to a precise definition of percentage of hemolysis, the especial value of a method such as the one here described becomes apparent. The kymograph tracings obtained with it are exact and unequivocal. There may be doubt as to whether a certain point on the record indicates 75 per cent or 78 per cent hemolysis, but the point itself is not in doubt. In most experiments what is desired is not so much to know how long it requires to reach, for example, exactly 75 per cent hemolysis, assuming that this expression has any precise meaning, but rather how long it requires under the chosen conditions to reach a point on the hemolysis curve which can be represented by some reproducible standard. This is possible with the present method with a high degree of accuracy.

Summary

1. A simple method is described by which it is possible to measure with a very satisfactory degree of accuracy the rate of hemolysis where the time involved exceeds approximately one second. If the duration of the experiment is ten seconds or more, a complete graphic record of the entire process up to an apparent degree of hemolysis of between 80 and 90 per cent may be obtained.

2. The method may also be used for the accurate determination of the relative numbers of erythrocytes in different suspensions and, assuming a satisfactory definition for the expression "percentage of hemolysis," for the rapid estimation of the latter, within the range most useful for experimental purposes, with an error of no more than one or two per cent.

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SOME EFFECTS OF $HgCl_2$ ON FERTILIZED AND UNFERTILIZED EGGS OF *ARBACIA PUNCTULATA*

LEIGH HOADLEY[1]

(From the Zoölogical Laboratory, Harvard University and the Marine Biological Laboratory, Woods Hole.)

In connection with a study of the fertilization reaction in *Arbacia punctulata,* the writer reported, in a previous number of this journal (Hoadley, 1923), some observations on the relationship between the concentration of salts of the heavy metals and frequency of cleavage in the egg of that form. In the course of these experiments it was found that mercury had certain effects upon cleavage which appeared to be indicative, though sufficient information was not available at that time to warrant any discussion of the phenomena involved. During the past summer, a study of these effects has been made. The results of this investigation will be presented below.

In a series of the heavy metals, $HgCl_2$ stands apart from the other chlorides tested in respect to its action on cleavage and membrane elevation. Thus it is found that there is no concentration of the mercuric salt which inhibits membrane elevation while permitting cell division. Quite on the contrary, the presence of the mercuric ion appears at certain concentrations, to favor membrane elevation, resulting in much wider membranes than is the case in sea-water alone. Cleavage, however, is completely inhibited in solutions of relatively low salt content. When a series of concentrations is tested, it is found that the inhibition of cleavage is associated with certain changes in the egg which are peculiar to mercuric chloride, not being identified with the action of any of the other salts studied. The definite nature of these changes is most evident in solutions of $HgCl_2$ between M/30,000 and M/50,000. These concentrations are from three to six times as great as those required to suppress cleavage completely in eggs exposed

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[1] This investigation was aided by grant from the Milton Fund.

to the solution from the time of complete membrane elevation on. I would like at this point to describe very briefly the general picture presented by eggs under such conditions.

Eggs of *Arbacia,* transferred to a solution of $HgCl_2$ in sea-water (M/40,000) five minutes after insemination in sea-water, show well-elevated membranes. The nuclear region is just becoming visible in the living egg in the form of a bright spot. The pigment is composed of very small granules which are evenly distributed throughout the cortical portion. After approximately thirty minutes the entire picture is changed, being very different from that found in the control eggs. The pigment is clumped at one side of the egg, where it is bulging outward as though the egg were cleaving into two parts, a larger one containing little or no pigment and a smaller containing all of the pigment which appeared in the cortical portion at the time of transfer. After 10 minutes in this condition, the pigment again becomes distributed throughout the entire cytoplasmic portion of the egg which proceeds to cytolyze. The somewhat clumped pigment loses its red color and fades materially early in the process. The pseudo-cleavage and the behavior of the pigment prior to and after the pseudo-cleavage and the accompanying cytoplasmic phenomena involved are of especial interest.

EXPERIMENTS

In the present study, as in that cited above, the egg of *Arbacia* was used. Solutions of $HgCl_2$ were made in sea-water from an M/10 stock solution in distilled water. The sea-water used in the dilutions had been standing in the room for a considerable period of time to bring it to the temperature of the air. Because of previous experience, attention was focused on the behavior of fertilized eggs in $HgCl_2$ concentrations between M/30,000 and M/50,000.

The description of the experimental results will be made according to increase in the concentration of mercuric salt in the solution employed. It should be borne in mind that the weakest, M/50,000, is a concentration which is three times as great as that necessary to completely suppress cleavage in fertilized eggs placed in it directly after complete membrane elevation. The experiments were performed in the following way.

Sperm and eggs were obtained from fresh *Arbacia* after washing the individuals and all instruments in running fresh water to kill adhering gametes, and then in sea-water to guard against any effects of hypotonicity. The eggs were obtained by straining broken ovaries through cheese-cloth. They were subsequently washed in three changes of sea-water to carry off body fluid and small pieces of the gonads.

The eggs were then examined to be sure none had membranes elevated and to ascertain the condition of the gametes. No batches of eggs were used which showed one per cent in germinal vesicle stages. With due precaution no eggs will be found with membranes elevated as a result of sperm infection or hypotonicity. The eggs were inseminated 20 minutes (ca.) after the removal of the gametes from the ovary. A sperm suspension, made by adding one drop of dry sperm to 25 cc. of sea-water having been prepared, 7 drops of this were added to 50 cc. of sea-water into which the eggs from a single female had been placed. One control of uninseminated eggs in sea-water, and one in the $HgCl_2$ solution were reserved for subsequent examination. Observations were then made to determine the fertility of the eggs. No batch with less than 98 per cent of the membranes elevated was employed. The eggs were then allowed to stand in sea-water until 5 minutes after insemination, when the transfer to the $HgCl_2$ was made. When insemination is carried out as stated above it will be described in the following way: 7(1:25):50.

The transfer of eggs to the $HgCl_2$ solutions and the retransfer to sea-water were accomplished as follows. Five minutes after insemination, 5 drops of the inseminated eggs were transferred to 10 cc. of $HgCl_2$ in sea-water and the eggs evenly arranged in the dish. This last precaution is very necessary in order to assure comparable results, for crowding affects not only the respiratory activities of the eggs, but also the concentration of the Hg^{++} ion in the immediate neighborhood, the initial amount being very small and the eggs containing substances which, as we shall see, are mercury-avid. The retransfers to sea-water were made at 3-minute intervals. One drop of eggs from the mercury solution was placed in 7.5 cc. of sea-water. All dishes were kept covered to prevent evaporation, and were also kept out of direct sunlight to prevent much change in temperature. The results obtained at any one concentration are so nearly the same that but one series of experiments will be described under each.

The information obtained in the present experiments may be presented in two ways, either of which would yield comparable data. We may consider each of the molecular concentrations of the salts, varying the period of exposure, or we may compare similar periods of exposure, varying the concentration of the solutions employed. Both methods of procedure have been employed. In general, however, the first method leads to greater simplicity, and will therefore be used in the description. A word should be said about the differences in response in batches of eggs from different females. This is, of course, to be expected, and might be satisfactorily explained could the relative

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amount of pigment be computed as well as allowance made for differences in the states of the gametes when used.

M/50,0000 $HgCl_2$.—This concentration was made up in sea-water from a M/10 $HgCl_2$ solution in distilled water. The temperature of the solution was 23° C. Eggs and sperm were prepared as described above and insemination made 7(1 : 25) : 50. Uninseminated controls in sea-water showed zero per cent membranes after 15 minutes. Inseminated controls showed 100 per cent membrane elevation 5 minutes after insemination. Uninseminated eggs in the $HgCl_2$ showed 0.5 per cent membrane elevation after 20 minutes' exposure. Five minutes after the sperm were added to the eggs, 5 drops of eggs were put into the M/50,000 $HgCl_2$ solution. This constituted the stock from which transfers were made to sea-water at 3-minute intervals up to 35 minutes. We shall now consider the effect of the solution on the eggs remaining in it, the changes on the return of the eggs to sea-water, the rate of cleavage in the eggs after each exposure, and the extent of their development.

Mercuric chloride of this strength has a very definite effect on the rate with which nuclear changes occur in the inseminated eggs. It is not as marked as in more concentrated solutions, but, over an extended period, e.g., 25 to 30 minutes, it is evident that the whole succession of nuclear phases is retarded. In this connection it should be mentioned that the effects are immediately made manifest in eggs in germinal vesicle stages (see Fig. 1 and note) and in eggs approaching the period of division. Save for the slight retardation, there is no

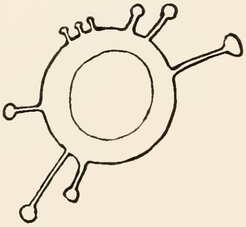

FIG. 1. Egg in the germinal vesicle stage showing the long processes expelled when egg is placed in the $HgCl_2$ solution.[2]

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[2] It is true that when *Arbacia* eggs are placed in sea-water a certain number of the eggs in the germinal vesicle stage will throw out processes similar in every way to those pictured here, but it is not true of all of the eggs in this condition. In the mercuric chloride solution it is the case with all of them. Whether this is a direct action of the solution on the more superficial regions of the egg or whether this is due to the indirect action of the salt on the membrane and subsequently the balance between the physical states of the cytoplasm and the medium is not clear.

evidence of any alteration of the eggs in the toxic solution until from 27 to 29 minutes, when 10 per cent of the eggs showed localization of pigment (cf. Fig. 2). With the increase in exposure time, the number of eggs showing the direct effect of the solution mounts thus, 32 minutes—30 per cent, and 35 minutes—40 per cent. With the localization of the pigment, cytolysis may be seen to be taking place around the clumped granules. The rest of the cortical portion of the egg remains intact. After long exposure, the intact eggs swell greatly, the contents being visibly coagulated and quite free of pigment. Fragmentation may be observed in some of the eggs in which pigment localization takes place. This is much more common in some of the experiments to be described below. No true cleavage ever is seen in eggs which remain in the $HgCl_2$ solution.

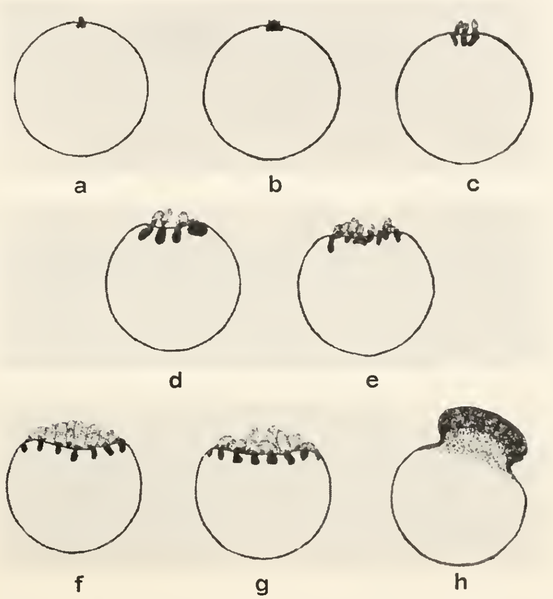

FIG. 2. Series showing the progressive effect that a series of exposure times has upon the localization of the pigment and the accompanying local cytolysis. In *h* may be seen the general picture presented by an egg undergoing "pseudo-cleavage." For explanation see text.

The effect of solutions of mercuric salt is not immediately apparent on the eggs. This is particularly noticeable in cases where the eggs are returned to sea-water after short exposures. It may be seen in the behavior of the pigment and subsequently in the rate and percentage of cleavage, as well as in the viability of the cleaving stages. It will be recalled that in the $HgCl_2$ solution, noticeable clumping of pigment appears at from 28 to 29 minutes. In the transferred eggs, however, some 3 per cent show a great deal of pigment localization with pseudo-cleavage at an age of 45 minutes, even when the transfer to sea-water has taken place after 18 minutes' exposure. Thirteen minutes later, these same eggs showed 55 per cent localized pigment. In eggs which were transferred after 15 minutes' exposure, while no pseudo-cleavage was found, 48 per cent showed localized pigment with cytolysis after 57 minutes. In eggs which have been exposed to the $HgCl_2$ solution for longer periods of time, the percentage showing pseudo-cleavage and localized pigment increases directly with the length of exposure. This last involves not only the extent of the action in individual eggs, but also the number of eggs affected. Generally speaking however, the extent of the phenomenon in individual eggs is directly comparable after similar exposure times. This varies slightly with different batches of eggs employed.

The extent to which pigment clumping takes place indicates a mechanism responding in a purely quantitative fashion. In Fig. 2 may be seen a series of effects produced in eggs on return to sea-water after successively longer periods of exposure to $M/50,000$ $HgCl_2$. The clumping itself is but the initial step in a series of changes which will be described here. Before the actual accumulation of the pigment takes place, there is little evidence of migration within the cytoplasm of the egg. In other words, the pigment which takes part in this action does not form larger masses which move through the cortex, but the massing occurs only at the point of final grouping. The result is that there is no apparent local depletion of pigment, but rather, a general depletion which takes place evenly over the entire cortex. After a short exposure this may be similar to that shown in Fig. 2, *a*, while after longer exposure it is more extensive, as shown in subsequent figures. As might be expected, with the increase in the amount of the pigment activated, the area of the cortical region involved is increased. The clumped pigment, however, is evidently destined to expulsion by the cytoplasm. As it reaches the surface in the particular area shown, it breaks through the membrane as globules. These immediately swell and undergo changes which are customarily described in eggs as cytolytic. The area of "cytolysis" is directly referable to that of pigment localization, the remainder of the cortex remaining intact. In extreme cases where exposure is long (e.g. 30 minutes), all of the visible pigment may be removed from the cytoplasm, and instead of a "local cytolysis" of the area involved, this area may be budded off as seen in Fig. 2, *h*. If this budding is extensive it may result in what I have called a pseudo-cleavage; if it is not as extensive, the budding may not be complete, the pigment may again enter the pigment-free portion, and cytolysis follow around each of the pigment masses. The most interesting observations associated with this action are, that pigment is affected evenly throughout the cortex; that it is eventually localized at one region of the cortex; and that it is eliminated at this point, the elimination being accompanied by cytolytic changes. It would be of great interest to determine at just what point in the cortex the accumulation of the pigment takes place. Is it in any constant relation to the polar axis of the egg? This is of even greater importance after a consideration of certain facts concerning the subsequent cleavage. There is, however, no definite evidence available on this point. An hypothetical determination might very easily lead to great error. This will be mentioned again in connection with cleavage.

Not all of the eggs which show the effects of mercury action fail to cleave. It has been mentioned above that no cleavage takes place in $HgCl_2$ solutions employed, so that we may confine our consideration to those eggs which have been returned to sea-water. Two sorts of data, both dependent on cell division, may be obtained from the experiment. The solution reduces the total number of eggs cleaving, and, in addition, it increases the length of the period between fertilization and cleavage. In the series already considered above, the percentage of cleavage drops constantly from 100 per cent after 9 minutes' exposure, to zero per cent after 24 minutes' exposure. There were only a few eggs which showed any cleavage after 21 minutes' exposure, and in those cases the retardation was very great, the time not being recorded because of the fact that all eggs were thought to be dead. The length of period required for the cleavage shows much variation, but the figures to be seen in Table I, for example, represent the time of

TABLE I

Effect of M/50,000 $HgCl_2$ on the time of the appearance of the first cleavage in eggs exposed to the solution for various periods of time. Time in minutes after insemination.

Exposure *minutes*	First egg observed in first cleavage	
0	42 minutes	
3	48.5 "	
6	50. "	
9	53. "	
12	55. "	
15	68. "	
18	71. "	
21	74. "	(attempted)

appearance of the first cleaving egg seen in each group of one batch. The retardation is marked. The interpretation of conditions within any one dish of eggs is further complicated by the fact that not all of the eggs which show the localized cytolysis around the pigment, cytolyze completely. In many cases in which cytolysis does not become more extensive than is shown in Fig. 2, *a, b, c,* and even *d,* the elimination of pigment is not fatal to the egg, which subsequently divides. This recovery, if it may be called such, appears to be more complete, and sooner complete, the less pigment is involved in the cytolysis. Thus, in one case, after an 84 per cent localized pigment cytolysis of the type shown in Fig. 2 *b*, 94 per cent of the eggs cleaved. Such behavior suggests the question as to possible relation between point of cytolysis and plane of cleavage, a point which I shall discuss here for all of the material considered.

There is a definite relation between the cleavage planes and the point of pigment accumulation and cytolysis. Whether this indicates an orientation of this point to polar orientation of the egg, or an effect of the localization of the pigment clumping on the orientation of the cleavage spindle, is not indicated in any of the experiments. In either case, the subsequent cleavages occur as shown in Fig. 3, *a, b,* and *c*.

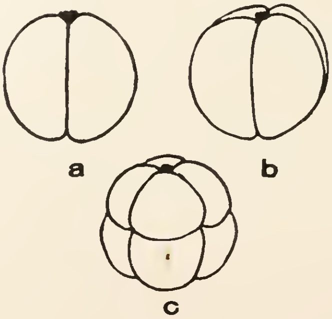

FIG. 3. Sketches to show the relationship between the point of pigment extrusion and the first, second and third cleavage planes. For explanation see text.

The relation of the extruded pigment is plainly visible because it remains attached to the blastomeres for a considerable length of time. All evidence obtained seems to point to the probability that this pigment is of relatively little significance as far as the early development of the germ is concerned. Cleavages follow as in typical development and motile larvæ are formed. A more complete discussion of this point will be found below.

The number of motile forms produced after exposure of eggs to the mercuric chloride also expresses the toxicity and inhibition already observed above. Table II is a record of conditions in the cultures 18 hours after insemination. Any exposure up to 9 minutes may be seen, on reference to the table, to have relatively little effect on future development. With the advance to 12 minutes' exposure, however, the number of viable individuals has dropped to 60 per cent, and these show marked retardation. After 15 minutes' exposure the retardation is even greater, as is also the case after 18 minutes. Viability also decreases. The possible relationship between the retardation and the action of the pigment will be considered below.

TABLE II

Effect of M/50,000 $HgCl_2$ on the rate of development of larvæ after exposure of the eggs for various times as indicated. The examination of the culture was made eighteen hours after insemination of the eggs.

Exposure *minutes*	Motile *per cent*	Extent of development
0	100	Young larvæ
3	100	" "
6	100	" "
9	90	" "
12	60	Very late gastrulæ
15	40	Early gastrulæ
18	30	Blastulæ
21	1	Early blastulæ
24	0	

M/45,000 $HgCl_2$.—As in the experiment just described, this concentration of $HgCl_2$ was made up in sea-water from an M/10 stock solution in distilled water. The temperature of the sea-water used in the dilution was 22° C. Eggs and sperm were prepared with the customary precautions. After controls of the uninseminated eggs had been set aside, the balance of the gametes were placed in 50 cc. of sea-water to which sperm suspension was added according to the formula 7(1 : 25) : 50. The eggs were all mature and showed 100 per cent membrane elevation 5 minutes after the addition of the sperm. The uninseminated eggs in sea-water showed zero per cent membrane 20 minutes later. No membranes were observed on uninseminated eggs in $HgCl_2$ 20 minutes after transfer. Five minutes after insemination, 5 drops of the eggs were added to 10 cc. of the M/45,000 $HgCl_2$ solution and these were evenly distributed on the bottom of the container. From this lot, samples were returned to sea-water at 3-minute intervals until 10 lots were available for study. The results of the observations follow.

The mercuric chloride in this concentration has a far greater inhibiting effect than in that dealt with in the previous section. This is noted first on observation of the nuclear changes in the egg. After about six minutes in the solution, the eggs show the monasters characteristic of the earliest phases and with this the progression of stages stops. If the eggs are observed at intervals one finds that gradually changes occur which concern the distribution of the pigment in the cortical cytoplasm, but these do not involve further changes in the nucleus. Those eggs in which the pigment does not clump gradually swell and eventually show a coagulated cytoplasm within a much bloated membrane. In by far the greater number of the eggs the swelling is preceded by a pigment clumping which is much more extensive in this case than in that previously described. After 24 minutes in the solution, about eight per cent of the eggs show a localization of the pigment which is not always confined to one spot as was the case in the weaker solution, but may be located at two or three regions, immediately below the egg membrane (Fig. 4, *a*). This action is associated in a very small number of the eggs with a pseudo-cleavage of the type described above, or with a constriction of the pigment-free portion of the egg such as may be seen in Fig. 4, *b*. The extent of the occurrence of such forms increases with the length of the exposure as does also the frequency of the eggs with multiple pigment centers, so that after some 40 minutes, when all of the eggs are swelling and none of them appear at all normal, some eggs may be found with as many as six of these

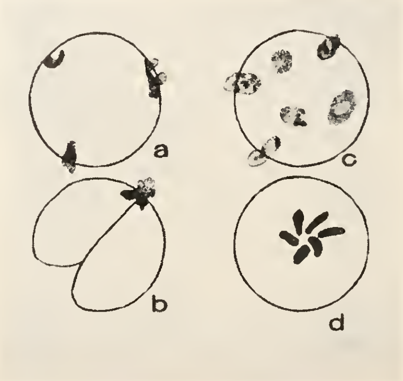

Fig. 4. Sketches to show more extensive action of the solution on the eggs. In *a* and *c* may be seen various degrees of multiple pigment foci. In *b* is an egg with purely cytoplasmic cleavage, a process resembling fragmentation. In *d* is a "polar" view of an egg to show the general picture presented by the clumped pigment. For explanation see text.

centers arranged as in Fig. 4, *c*. These forms are always much rarer than those of the pseudo-cleavage type.

It is not as easy to follow the course of the pigment clumping in eggs in this concentration as it was in the previous one. This is true for two reasons. A difference of 3 minutes in the exposure time in the higher concentration is equal in its effects to that of a 12-minute interval in the lower concentration. Moreover, the pigment changes occur very rapidly after the eggs are returned to the sea-water, and the duration of the intermediate stages is shortened. Hence their determination is rendered more difficult. Associated with this more rapid reaction within the egg after its return to sea-water, we find an increase in the number of eggs in which the clumping of the pigment is complete. Thus there is a rise to 20 per cent of pseudo-cleavage in eggs that have been exposed to the mercuric solution for 24 minutes. This complete isolation of the pigment appears for the first time in eggs which have had a 15-minute exposure to the solution. Some effect on the pigment is found even in the 6-minute eggs. It is not extensive, resembling in most of its details the picture presented by the eggs in Fig. 2, *a*. Of these eggs, 40 per cent were so affected while 98 per cent of them cleaved. In the 9-minute eggs the condition is much more advanced. Pigment clumping is accompanied by local cytolysis as in the previous cases. The situation is very much more general here, being found in 96 per cent of the eggs. There is a 100 per cent local cytolysis in the 12-minute eggs which is far more extensive in its nature than anything described in the discussion of the first series. A rather typical egg may be seen in Fig. 4, *d*. Further exposure tends to produce even more extensive clumping of the pigment, with pseudo-cleavage, and with the multiple pigment centers. These last are found in eggs which appear for the most part to have a coagulated cytoplasm. It looks very much as though pigment localization having been initiated, coagulation had set in so that migration was stopped, leading to pigment clumping around a number of centers. Local cytolysis occurs around each of the pigment masses and swelling follows. None of these eggs ever show any further developmental changes.

The effect of the $HgCl_2$ on cleavage is much more extensive in these concentrations than in the weaker ones. Immediately following the retransfer of the eggs to sea-water, there is a rapid change in the nuclear components, so that bar and streak stages are formed. This takes place only in those eggs which have been in the solution for 15 minutes or less, though an occasional egg is found which will proceed after 18 minutes in the toxic solution. There is a progressive retardation as the length of exposure increases. Thus, the 3-minute group cleaved 9 minutes after the controls, while the 15-minute group was retarded 75 minutes. In the 18-minute group, only an occasional egg cleaved, and then 135 minutes after division in the controls. The effect may most readily be seen in the percentage of cleaving eggs. This drops from 98 per cent after 3 and 6 minutes' exposure, to 90 per cent after 9 minutes', 72 per cent after 12 minutes', 18 per cent after 15 minutes', and less than one per cent after 18 minutes'. The percentage of cleavage is, therefore, inversely proportional to the length of the exposure to the mercuric solution. Inasmuch as there is a 40 per cent local cytolysis in the 6-minute eggs, 96 per cent in the 9-minute eggs, and 100 per cent in the 12-minute eggs, it is evident that many of those which have shown local cytolysis must cleave. This has been observed here as in the cases described above. The attached extruded cytolysing pigment can be traced through at least the 16 and 32-cell stages, as was the case above (cf. Fig. 3). Additional information on viability is available for these eggs, however, for observations were made periodically up to the time of pluteus formation.

Motile larvæ were produced by cultures of eggs removed from the $HgCl_2$ solution after 3, 6, 9, and 12 minutes' exposure. Of these, only the first three continued in their development to the formation of plutei. Forty-eight hours after insemination in the first two cultures, *i.e.,* in those of 3 and 6 minutes' exposure to the solution, the plutei produced appeared typical in every way. In the 9-minute culture, on the other hand, the few plutei formed were much retarded. The arms were short and the body heavy. A relatively small number of the larvæ continued their development to this point. It will be recalled in this connection that before cleavage, 96 per cent of these eggs showed localized pigment with an associated cytolysis which was quite extensive. In the 6-minute group, the localized cytolysis and pigment clumping had affected 40 per cent of the eggs. While a definite statement cannot be made about the 9-minute culture, it was perfectly evident that over 60 per cent of the 6-minute eggs had formed plutei. The experiment shows that typical plutei can be formed from the eggs which have undergone the pigment localization and localized cytolysis produced by short exposure to M/45,000 $HgCl_2$ solutions.

M/40,000 $HgCl_2$.—The temperature of this concentration, which was made up in sea-water from the M/10 stock solution of the salt was 21.3° C. Eggs and sperm were prepared as before and insemination carried out according to the same formula, 7(1 : 25) : 50. The usual controls of uninseminated eggs were set aside. That in sea-water showed no membranes elevated after a period of 30 minutes. In $HgCl_2$ solution, examination of the material showed 8 eggs with wide membranes (less than one per cent) after 15 minutes' exposure. Inseminated controls showed 100 per cent membrane elevation after 5 minutes and 100 per cent cleavage at 45 minutes. Five minutes after insemination, 10 drops of eggs were added to 10 cc. of the $HgCl_2$ solution. These were returned to sea-water at 3-minute intervals until 10 transfers had been made. The results of the experiment are similar in many ways to those already described above, save that the modifications in the eggs appear earlier. After longer exposures, there are some differences which will be discussed below.

The transfer of the eggs to the $HgCl_2$ solution is immediately followed by an increase in the width of the space formed by the elevation of the membrane. Results of a similar nature were obtained in previous experiments already referred to in the introduction. The increase is much greater than any noted at weaker concentrations. In the meantime, the nuclear elements appear as monasters. This is as far as the nuclear changes go in the solution, no bar or streak being formed. The eggs remain in this condition until the total exposure amounts to approximately 24 minutes, when the pigment begins to show the clumped effect typical of longer exposures in less concentrated solutions. Subsequently the eggs appear to be damaged rapidly, swelling and coagulation being evident in a large percentage. The most marked effects appear in the eggs which have been transferred to sea-water after exposure to the $HgCl_2$.

There is little evidence of any modification in the development of eggs exposed to the solution for three minutes save in a slight increase in the length of time elapsing between insemination and the first cleavage. This does not markedly reduce the viability of the individuals over a long period of time, however, for most of them continue in their development and form subsequent stages at approximately the same rate as the controls. After 6 minutes' exposure, the results are essentially the same save that but 90 per cent of the eggs form blastulæ, a reduction of 6 per cent of the total number cleaving. The viability is good. Nine minute eggs show a marked effect which is evident first as the localized cytolysis described above, and later in the reduction of the percentage of cleaving eggs. Subsequently, further evidence of the solution's action is seen in the retardation of development and in the lowered viability of eggs in later cleavage stages. In many, development stops after the first, second, or third cleavage. The percentage of dead individuals in the cultures is continually increasing. As a result, relatively few reach even the early gastrula stage. These few show a marked retardation when compared with the control embryos or with those of cultures of 3 and 6 minutes' exposure.

The condition just described is much further developed in the 12-minute eggs. At least 10 per cent show a local cytolysis which involves a greater portion of the cortical region than is concerned in the 9-minute eggs. Cleavage is retarded far more and, when some of the eggs finally do cleave, the total number is but 20 per cent, whereas it was 96 per cent after 9 minutes' exposure. The lower percentage of the cleaving eggs is apparently related to an increase in the amount of the pigment clumping, which here shows the form pictured in Fig. 4, *a, c,* and *d.* Several eggs had cleaved in the cytoplasmic portion (cf. Fig. 4, *b*), a phenomenon much more frequently encountered in cultures of eggs exposed for longer intervals. It will be described in more detail below. In addition, some of the eggs show the isolation of the pigment as in Fig. 2. Examination of the culture after 24 hours showed but an occasional embryo still living, and in those development had been very atypical.

The analysis of material removed from the toxic solution after longer exposures is greatly complicated by the appearance of more of the type of egg shown in Fig. 4, *b* than were found in the 12-minute group. In addition, eggs are frequent in which the pigment-free cytoplasmic portion is divided into even more units. Some of these are shown in Fig. 5, *a, b, c,* and *d.* It is always accompanied by the

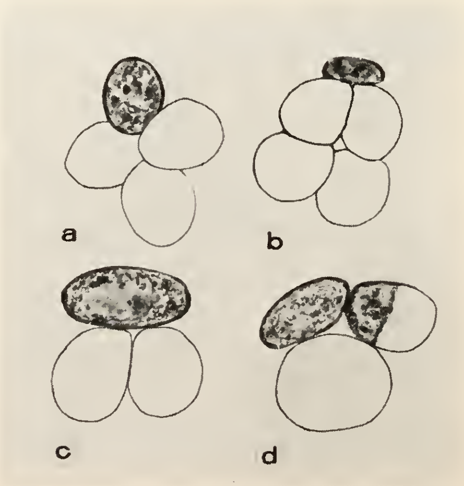

FIG. 5. Drawings to show the relation between the completely isolated pigment and the cytoplasmic fragmentation or cleavages which are found after exposures to more concentrated solutions of the salt. The cleavage in such cases does not involve the nucleus. For explanation see text.

accumulation of all of the pigment in one region of the egg. In the initial stages it looks much like a typical cleavage but, inasmuch as it is followed by coagulation and swelling and nuclear changes are not visible, it is evidently not a true cleavage but rather a purely cytoplasmic phenomenon. This conclusion is confirmed by the appearance of the eggs after fixation. The number of the eggs undergoing typical division falls abruptly. This may be seen in Table III. Many readings of the eggs have to be made to separate those with the cytoplasmic division from those showing true cleavage following rather extensive pigment localization. Approximately 50 per cent of the eggs show the cytoplasmic division. The majority of the remainder show the type of pigment localization pictured in Fig. 4, *c*, though there may be many more of the isolated pigment masses than are shown there. In the 10 per cent (ca.) of the eggs which eventually undergo a true cleavage the viability is very poor. As a result, less than one per cent go as far as the 32-cell stage and these die shortly afterward. Death is accompanied by swelling and evident coagulation of the cytoplasm.

Table III

Effect of $M/40,000$ $HgCl_2$ *on rate and percentage of cleavage of eggs after various periods of exposure. Readings taken 227 minutes after insemination. Where the exposure is more than six minutes the viability of the eggs is poor.*

Exposure *minutes*	Cleaved *per cent*	Extent of development
0	100	64-cell
3	98	64-cell
6	96	90% 64-cell
9	94	32-cell (many dead)
12	20	8-cell (cyt.)
15	4 (ca.)	Complicated by cleavages as in Fig. 4, *b*.
18	0	
21	0	

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$M/35,000$ $HgCl_2$.—Experiments with this concentration of the $HgCl_2$, when compared with those of less salt content, serve for the most part to demonstrate the increased toxicity of the solution on eggs after shorter periods of exposure. The solution was prepared in the same way as those above. Controls were set aside for future examination. Eggs were obtained from one female and sperm from one male. The insemination was made according to the formula, 5(1 : 25) : 50. The uninseminated control in sea-water showed no membrane elevation after 20 minutes while the uninseminated control in the $HgCl_2$ solution showed a little less than one per cent after but 7 minutes' exposure, a percentage which increased with the length of

exposure. The inseminated eggs showed 100 per cent membrane elevation at the end of 5 minutes, when transfer to the toxic solution took place. In the mercuric solution there was a noticeable widening of the cortical space between the fertilization membrane and the egg. The nucleus forms the monaster, but no further nuclear changes take place. As in previous cases, transfer to sea-water followed at 3-minute intervals. For the most part the results obtained in the experiment are directly comparable to those already described above, save that the action of the salt is evident after shorter exposure times. The greatest difference is seen in the occurrence of eggs with cytoplasmic division after as short an exposure as 18 minutes, and the relatively large number of eggs with multiple pigment loci. In this concentration, the effect is quite evidently one of cytoplasmic coagulation. The description of the first four groups of eggs, *i.e.*, those exposed for 3, 6, 9, and 12 minutes respectively deserves especial mention.

There is no localization of pigment in any of the eggs which have been in the $HgCl_2$ solution for but 3 minutes. One hundred per cent of the eggs cleave regularly, though in this experiment the cleavage takes place after a period of 53 minutes rather than 42 minutes after insemination, as in the controls. No other effects of the exposure appear up to the time of larva formation. The cleaving eggs go through the early developmental stages but slightly retarded, and form motile larvæ similar to the controls. This is not true of the eggs which have been exposed for 6 minutes. This group shows a marked localization of the pigment followed by extensive local cytolysis, (94 per cent of the eggs at 60 minutes). Quite a large amount of the cortical region of the egg is involved. The condition resembles that shown in Fig. 2. In spite of this 98 per cent of the eggs cleaved, though the cleavage did not start in any of the eggs for 63 minutes, 10 minutes later than division in the 3-minute culture, and 21 minutes later than the controls. The deleterious effects of the exposure are evident in each of the following exposures. Three and one-fourth hours after insemination, when the eggs of the control and the 3-minute group are in 16 and 32-cell stages, this culture shows not only 16-cell stages but also 2, 4, and 8-cell stages. Fifty per cent of the eggs are alive, while the rest have cleaved once, twice, or three times, where the development stopped. None of the eggs develop motile larvæ. It is quite evident, therefore, that the viability of the eggs has been markedly decreased by this short exposure to the action of the mercuric salt.

Quite in contrast to the rest of the experiments described, some few of the eggs which were in the 9-minute group show a pseudocleavage of the type pictured in Fig. 2. In no other case is this evident after such short exposure. Localization of pigment takes place in all of the eggs after 60 minutes and in the majority is to be found in numerous foci (vid. supr.: multiple localization). The few eggs not showing either of these phenomena retained, theoretically at least, the possibility of dividing. No cleavage was observed in these 3 hours after insemination. Fifteen minutes later approximately 2 per cent of the eggs had divided. The viability of these few was so diminished, however, that none of them went beyond the 4-cell stage. The poisoning is complete in the 12-minute eggs, where no division takes place. This is probably intimately associated with the increase in the percentage of pseudo-cleavages and the marked extensive local cytolysis which takes place immediately upon the return of the eggs to the sea-water. All of the eggs eventually swell and cytolyze, though there is a marked differential effect in different eggs.

M/30,000 $HgCl_2$.—This experiment was set up in the same way as those described above. Uninseminated controls showed no membranes after 20 minutes when in sea-water, and over one per cent wide membranes when in $HgCl_2$ solution. One hundred per cent membranes were elevated on insemination and this was followed in sea-water by cleavage at 46 minutes. The temperature of the solutions at the initiation of the experiment was 22° C. There are a number of ways in which the eggs here differ from those described in former experiments, some of which involve merely an intensification of a previously noted action of the salt, while others introduce new phenomena. The first indication of the effect of the solution appears as a clumping of the eggs. This has not been noted in any of the previous experiments. There is evidently a modification of the quality of the surface membrane of such a nature that the eggs adhere to each other. Some modification or solution of the jelly layer investing the egg must be involved, for control eggs showed the jelly layer. This is followed by a pseudo-cleavage in the mercuric solution after 12 minutes' exposure. Such eggs cytolyze at the point of pigment accumulation after 3 to 6 minutes. After 30 minutes 80 per cent of the eggs show a cytolysis of the cortical region associated with an accumulation of pigment. The eggs transfered to sea-water show much the same sort of behavior. Even in eggs which are transferred after 3 minutes' exposure there is 100 per cent local cytolysis 63 minutes after insemination. Much of the cytolysis is very extensive. The pigment accumulation in the region of the cytolysis is a little greater than that described above for 6-minute eggs. Eggs which have been exposed to the mercuric solution for longer periods of time show further effects of the action of the salt. In many, coagulation appears very early, so that further cytoplasmic changes and pigment migration stop, the eggs merely swelling and cytolyzing. Some of the most interesting modifications take place in the uninseminated eggs which were placed in the $HgCl_2$ solution. The pigment is clumped, but in a way visibly different from that in inseminated eggs. In addition, there is a cytoplasmic cleavage or fragmentation which is partial, involving one side of the egg only. This appears in but a very small percentage of the eggs. It may be associated with those in which membrane elevation has been produced by the solution, though no direct evidence on this point is available. No nuclear changes are involved.

In previous experiments nuclear changes proceed after inseminated eggs which have been in $HgCl_2$ solutions are returned to sea-water. It will be recalled that in the more concentrated solutions, the nucleus remains in the monaster stage during the time that the egg is in the toxic solution, further progress appearing only after retransfer. The nuclear changes in the solution of M/30,000 concentration proceed very slowly to the monaster stage, even this change occurring only in a small percentage of the eggs which are not greatly effected, after 18 minutes in the solution. When these are returned to sea-water, the changes proceed very slowly. Only those eggs which have been exposed for but 3 minutes show any advance over the monaster. These eggs are greatly retarded, showing only an early bar 27 minutes after insemination. The evidence of extensive injury is marked, therefore, not only in the behavior of the cytoplasm and its inclusions, but also in the nucleus. The depression of the viability of the eggs is so great in this experiment that none of the eggs divided, even when the exposure is as short as 3 minutes. This may be associated with the relation between nuclear and cytoplasmic phenomena.

Discussion

From the results obtained and presented above, we find that the mercuric chloride solution produces several specific changes in the egg, all of which vary directly with the concentration of the solution and the length of the exposure. A number of these phenomena deserve special consideration in the discussion. The "activating" influence of the $HgCl_2$ at certain concentrations will concern us first.

It has previously been reported (Lillie, 1921; Hoadley, 1923) that in relatively high concentrations of mercuric chloride, not only is the elevation of membranes wider on inseminated eggs than on inseminated sea-water controls, but uninseminated controls show a certain percentage of eggs with membranes. The percentage of uninseminated eggs elevating membranes on exposure to the solution varies directly with the concentration of the salt. The production of the membranes is not immediate. They appear on certain eggs after as short an exposure as five minutes, in others the membranes do not elevate for a much greater period of time. There is no possibility of either hypotonic or hypertonic activation in any of the experiments cited. Although the subsequent development of eggs showing membranes elevated after exposure to mercuric chloride solutions has not been investigated, this must, I think, be interpreted as an initiation of development in the gametes. The evidence for this statement is derived entirely from close observation of the pigment behavior and the subsequent cytolysis of such eggs as compared first with that of membraneless eggs, and second with that of eggs with fertilization membranes elevated before exposure, both lots being left in the mercuric chloride solution during the observations. The eggs with membranes elevated after exposure to the $HgCl_2$ resemble the inseminated eggs in every way. There must be some alteration of the cortical membrane of the egg induced by the solution which is similar to that produced at fertilization, and which results in the elevation of a membrane and, subsequently, the like penetration of the mercuric ion. As a result, the reaction to the $HgCl_2$ is that of an inseminated rather than of an uninseminated egg.

As would be expected in the case of an extremely toxic solution, the effect is next evident in the cortical cytoplasm of the egg. The pigment granules are visibly affected first and most extensively. This in turn appears to be due to a specific mercury-avid property of the pigment itself. The degree to which the total amount of pigment in the egg is involved in this action is apparently of a quantitative nature as is evidenced by the experiments. Similar effects are produced by short exposure to concentrated solutions and longer exposures to less concentrated solutions. An indication of the extent of the action may be obtained by examining the behavior of the pigment subsequently accumulating at the point of the egg at which extrusion and local cytolysis occur. The very fact that after the extrusion of the pigment which is localized, as aforesaid, cleavage may take place and larvæ may be formed indicates that the cytoplasm as a whole is not greatly affected by short exposure to the mercury. Longer exposure has a more extensive effect, so that coagulation appears and the eggs are much damaged, further development not taking place. If, as would seem legitimate, one may regard the pigment as a mercury-avid substance, a conceivable mechanism is available by which the mercuric ion which has entered the egg may be bound and removed. Hence the egg is enabled to continue its development. The clumping of the pigment is very evidently not primarily a local response to the presence of the mercuric salt. Rather, as is evident in many of the experiments, the pigment is equally affected throughout the entire cortical region of the cytoplasm. At a later time, the pigment becomes clumped or localized at some single point near the surface of the egg. Where the pigment clumps in a single mass, no coagulation of the egg cytoplasm appears, as is the case on exposure for longer periods of time or to more concentrated solutions. Where coagulation takes place before the migration of the pigment is complete, there are a number of small foci, isolated one from the other. Apparently the migration of the pigment has been arrested by the coagulation of the surrounding cytoplasm. It is also interesting to note that in the majority of cases where coagulation does not occur, cleavage may follow the expulsion of the single pigment focus. There is no evidence of any correlation between the site of pigment accumulation and the original polarity of the egg in *Arbacia*. It is possible that an examination of the behavior of other forms may yield valuable information concerning this point.

The elimination of the pigment in the eggs and their continued development is of interest from yet another standpoint. Pigment in eggs has been regarded as of developmental significance by many workers. By some it has been thought to be of importance because of its association with oxidative processes within the germ. Warburg (1914), in experiments with *Strongylocentrotus lividus,* found that oxidations in the egg were associated with the granular portion. We might expect that if oxidative processes are intimately associated with the granular portion of the egg, and hence, in part at least, with the pigment granules, loss of the pigment granules would have a definite effect on the future development of the egg. It has already been shown by a number of workers that oxidative processes and developmental rate over short periods of time are discrete in their action. This is emphasized by Whitaker (1929) in a report of observations on the relative rate of development in pigmented and unpigmented fragments of *Arbacia* eggs. In the experiments reported above there is an evident retardation in the developmental rate and also a marked reduction of the viability of the gametes. In view of Whitaker's results, however, we must look in another direction for the interpretation of the facts.

The retardation in developmental rate is evident in all cases in which eggs are exposed to mercuric chloride solutions. The extent of both the slowing down of the cleavage rate and the loss of viability is directly dependent on the strength of the solution and the length of the exposure. Apparently it is more extensive after pigment has been extruded than when it has not, but this is always attendant on a longer exposure period or a greater concentration of the solution. There is a perceptible lag of the effect on the viability over the effect on the rate of development. They show a correlation in that they both increase with an increase in the effective period of the exposure. Eventually the interpretation of the two is confused by the fact that only one or two cleavages take place, the individuals then dying. This is especially marked after even short exposures to high concentrations of the salt. The amount of the pigment extruded in such cases is not very much greater than that at lesser concentrations, and besides, we are dealing with the early stages in development when oxidation rate and developmental rate are quite independent. For that reason it appears that the mercuric ion, both in retarding development and in lowering the viability, acts directly on the cytoplasm of the egg rather than indirectly, through the medium of the pigment granules.

Two types of cytolysis are associated with the action of the mercury on the egg. In all cases where the eggs are allowed to remain in the solution, they eventually coagulate and swell, no cleavage taking place. The picture in the eggs which are retransferred to sea-water is quite different. There, the pigment accumulates and the cytolysis which follows involves only the region of pigment clumping and extrusion. The rest of the cytoplasmic portion of the egg is little affected, as has been mentioned above, so that cleavage and larva-formation follow. The phenomena involved in the local cytolysis associated with the pigment elimination lead to the conclusion that the product of the mercury bound pigment is responsible for this action. The mechanism involved is not clear.

The results of the investigation may be briefly summarized as follows:

1. Mercury has an effect upon the egg of the sea-urchin *Arbacia* which is unlike that found in the case of any of the other metallic chlorides investigated.
2. Acting first on the cortical region it activates membrane elevation.
3. After longer exposures it has a direct effect upon the pigment which has mercury-avid properties. The pigment reacts to the mercuric solution by accumulation and subsequent extrusion at a localized point (or points) on the surface of the egg.
4. The extrusion of the pigment is accompanied by a cytolysis of the pigment granules and the associated cytoplasm.
5. The development of the zygote is retarded and its viability is lowered by the action of the mercuric solution.

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THE CLEAVAGE OF POLAR AND ANTIPOLAR HALVES OF THE EGG OF CHÆTOPTERUS

DOUGLAS WHITAKER AND T. H. MORGAN

(From the Marine Biological Laboratory, Woods Hole and the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology.)

The following experiment was carried out as part of a program to study the phenomenon of yolk-lobe formation that occurs in the eggs of certain annelids and molluscs. This peculiar phenomenon simulates cell-division to a striking degree, yet at the final moment, when the yolk-lobe is attached only by its stalk to one of the cells, a reversed reaction takes place and the material of the lobe is absorbed by the egg, or by the blastomere to which it is attached.

There were two relations that we wished to examine by cutting the egg in two: First, to find out whether the polar lobe develops on both fragments or only on one of them; second, whether the unequal first cleavage is dependent on or the result of the presence of an antipolar yolk field, or independent of its presence. In addition there were one or two other questions that we hoped to clear up: *e.g.,* whether there is any relation between the appearance of the pear-shaped form that the undivided egg assumes just prior to the appearance of the yolk-lobe and the mitotic phenomenon; whether the surface of the egg in the region of the constriction that produces the yolk-lobe changes before or during the formation of the lobe; and whether the condition of the mitotic figure at the moment of the lobe's formation bears any causal relation to the formation of the lobe. The experiments were made in the summer of 1928 at Woods Hole.

Eggs taken from parapodia were put into sea water. The germinal vesicle breaks down in a few minutes, and a spindle, pointing to the pole, is formed. The polar region can be made out owing to the clear region around the spindle. The egg remains in this condition until fertilized. Just before cleavage the egg becomes pear-shaped with the apex at the pole. A little later the egg begins to bulge at the antipole to form the yolk-lobe that does not constrict off from this egg as markedly as it does in some other eggs, such as *Ilyanassa* and *Dentalium.* Sections of the preserved egg during these periods show that the pear-shaped stage first appears when the two pronuclei have come together and the walls are disappearing. The two asters of the future division-figure are present and well developed. Just what internal condition leads to the change in shape is not shown from these relations. One might surmise, however, that the collapse of the very large pronuclei with the resulting distribution of their fluid contents, or possibly the nearer approach of the two astral fields resulting from the collapse of the nuclei, may be connected with the change in shape of the whole egg.

Almost immediately the egg becomes rounded again and begins to elongate in a direction at right angles to the plane of the oncoming first division. At this time a bulging around the antipole indicates the development there of the antipolar lobe. As the cleavage proceeds the lobe becomes more conspicuous, and later becomes constricted at the point of contact with the egg. The constriction becomes deeper, giving the lobe an oblong or even rounded appearance, but in *Chætopterus* the constriction is never carried as far as in the eggs of some molluscs. At this time the cleavage furrow is progressing, but, from the first, does not give so much the appearance of cutting through the lobe as in *Dentalium*. As the furrow deepens it passes to one side of the lobe. The point of attachment of the lobe remains on the larger or CD-blastomere.

Sections of eggs that have been preserved in picro-formalin, or Flemming's osmic acetic, stained in iron hæmatoxylin and counter-stained in erythrocene, do not reveal any unusual changes in the anti-polar region during this period. Some of the yolk (and its surrounding protoplasm) simply protrudes into the lobe. This yolk is a part of the cup-shaped mass that lies over the lower hemisphere with the edges of the cup extending toward the polar field. The yolk that goes into the lobe is not discontinuous with the rest of the material. One gets the impression that it is squeezed into the protrusion or bulges into it as the lobe develops. As the base gradually constricts there is, in sections, visible in the superficial layers nothing that is peculiar or different from the rest of the neighboring surface. The impression that one gets from sections is that the rounding up of the materials that become the two blastomeres does not include the antipolar field, and that the lobe is a by-product, so to speak, of these changes, and not in itself actively engaged in the process of its formation.

This interpretation may appear at first sight to be in contradiction to the observation that Wilson has made of the behavior of the yolk-lobe of *Dentalium* when severed from the CD-blastomere. He found that the isolated lobe showed alternate periods of rest and activity that were synchronous with those of the next two divisions of the egg when the yolk-lobe reappeared. In some cases he observed indications that the yolk-lobe itself formed a lobe. These observations may not appear to harmonize with the supposition offered above, that the lobe, as such, is a passive factor in the result, and its development the resultant of the mitotic constrictions about the division centres; but if, as is not impossible, a rhythmical impetus, or something of the sort, is initiated in the cytoplasm, it might conceivably be supposed to affect even the isolated lobe, or even involve the formation of a cytoplasmic aster in the lobe. Until observations are made on the interior of the isolated lobe at the time of its activities we can only speculate as to the causes of this remarkable phenomenon.

The operations on the egg were made before fertilization. The outer membrane is very tough, even before fertilization, so that it is difficult to sever it completely without destroying the egg within. This difficulty is increased by the tendency of the *Chætopterus* egg to burst, and care is necessary in cutting in order to avoid great injury. It can be done, however, with a quartz needle and the micro-dissection instrument. The egg is least injured if slowly pinched apart, and if the membrane is gently twisted by rolling the needle back and forth. Damage to the fragments is further reduced if the outer membrane is not entirely severed after the egg is well cut apart. The results obtained from a few eggs with membranes entirely severed were the same as those obtained from the eggs whose fragments remained in the same membrane. It does not seem likely that the membrane connection between the fragments has any effect. Independent fertilization of both fragments takes place. All of the operations here described were made either in the equatorial plane or parallel to it.

The polar fragment becomes pear-shaped at about the time after fertilization when the egg passes through this stage. It then elongates for the first cleavage, but a yolk-lobe does not appear at the antipolar surface of the fragment. It is true that sometimes a bulging, or other irregularity, appears in the region where the cut was made, especially in the fragments most damaged in cutting, but it does not have the distinctive shape of the normal lobe. It seems rather to be due to some weakness at the cut surface, and as a result, perhaps, of changes of tension within the egg or at the surface.

The first cleavage of the polar fragment is into unequal parts, which, in general, have the same relative size as have the first two cells of the normal egg. Since the yolk region has been removed, and still the unequal cleavage appears in the fragment, the inequality cannot be explained in the normal egg as due to the presence of yolk or yolk-lobe material in the antipolar hemisphere.

The antipolar half of the egg, which contains only a sperm-nucleus, does not assume a pear-shape prior to the first cleavage, but at the time when the cleavage is about to begin a typical antipolar lobe appears. The size of the lobe is approximately proportionate to the size of the fragment. If at the time of the operation all of the material that normally goes into the antipolar lobe is already present in the antipolar hemisphere, it would appear that the formation of the antipolar lobe is not simply the extrusion of a given amount of inert material around the antipole, but is correlated with the size of the dividing materials and possibly with the size of the astral spheres.

The first cleavage is into unequal cells. The antipolar lobe comes to lie in the larger cell into which it is later absorbed. Whether this difference in size of the first two cells is in the same ratio as in the polar fragment cannot be stated positively, but there is certainly no striking difference in the two cases. Here, again, it may be pointed out that the unequal cleavage is not dependent on the amount of yolk in the dividing cell. Were it so, the smaller cell might be expected to be disproportionately smaller in the basal fragment.

These general statements may be supplemented by the following numerical data. In 19 cases both halves developed and conformed to the above description. In 3 cases the polar half conformed; the antipolar was somewhat abnormal. In 3 cases both approximately conformed but were somewhat abnormal. In 5 cases the polar conformed, the anti-polar did not develop; in one case neither developed. In addition there were two cases in which the antipolar lobe was not seen on the basal half, and in one case the antipolar half formed a lobe but did not divide; the polar half failed to develop.

Mead ('98) described the early cleavage stage of the egg of *Chætopterus* with numerous drawings of the mitotic figure during cleavage. At the time when the yolk-lobe appears, the " rays " from the astrosphere of the CD-cell are represented as extending into the polar lobe. The antipolar cleavage plane is represented as lying at the crossing-point of the rays of the two blastomeres to one side of the lobe. From the figures, which appear partly schematized, it is not certain whether these rays are at their outer ends anything more than lines resulting from the arrangement of the peripheral yolk granules.

Lillie ('06) has also described the early cleavage stages of the *Chætopterus* egg. He found that after centrifuging the yolk is driven away from the antipolar field in some of the eggs that have fallen in the centrifuge with the polar hemisphere turned outward. When these eggs cleave the yolk-lobe may contain the oil field, or the clear middle zone, proving that the yolk as such is not essential to the formation of the polar lobe. The more superficial layers of the egg are little, if at all, distributed by the centrifuging, and what is here more to the point, the mitotic figure occupies the normal position in such eggs with respect to the pole and to the antipolar field.

Wilson ('29) has recently described the cleavage and development of egg-fragments of the *Chætopterus* obtained by centrifuging. The eggs, for the most part, seem to fall at random in the centrifuge tube, and are stratified without regard to the polar axis—at least, all possible relations may be found. Strong centrifuging causes the eggs to elongate and often the clear (centripetal) end constricts off from the yolk-bearing end. The nucleated fragment, that lies nearer to the pole, can be identified because such a fragment gives off the polar bodies. Unless it could be shown that the spindle is also displaced at times by the amount of centrifuging here used, this result makes the identification of the polar fragment certain, regardless of whether it contains the yolk or the oil cap. After fertilization both fragments may cleave. Wilson finds that the unequal first and second cleavages are characteristic of both fragments. In general, only those fragments that do not give off polar bodies develop a yolk-lobe. Our own results confirm entirely these conclusions. Their only merit is that they give, perhaps, more accurate information regarding the regional origin of the fragments, and make possible comparisons between fragments of equal sizes of the same egg whose interior has not been disturbed by centrifuging.

If, then, as these experiments appear to indicate, the antipolar lobe-formation is not an essential part of the cleavage pattern but a by-product of that pattern, its absence from the polar fragment and its presence in the antipolar fragment remains to be explained. It is reasonably certain from Lillie's and Wilson's centrifuging experiments that this lobe is not directly caused by the presence of a particular kind of yolk material at the antipole, but the occurrence of this material might, by influencing the location of the mitotic figure, determine the extra-territorial region that becomes the lobe. If so, the relatively greater development of the mitotic figure in the polar fragment might in itself account for the absence in it of a lobe at the two-cell stage. Conversely for the antipolar fragment. But there is an alternative possibility, namely, the relative location (and size) of the first spindles in the two cases. If, for instance, it could be shown that in the polar fragment the spindle is relatively nearer the centre and in the antipolar fragment relatively nearer the polar side of the fragment, the two results would be in accord with the hypothesis suggested above.

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THE ABSORPTION SPECTRA OF SOME BLOODS AND SOLUTIONS CONTAINING HEMOCYANIN

ALFRED C. REDFIELD

(*From the Department of Physiology, Harvard Medical School, Boston and the Marine Biological Laboratory, Woods Hole*)

The present study of the absorption of light by hemocyanin was undertaken in the course of developing a spectrophotometric method for the determination of the quantity of oxygen combined by the blood of invertebrates which contain this pigment. The data obtained provide a precise description of the color characteristic of the body fluids of the various animals examined. Attention has been directed not only to the spectrum of oxygenated blood, which has already been examined with precision in the case of a number of organisms by Dhéré and his collaborators (1919, 1920, 1929), Begemann (1924) and Quagliariello (1922), but also to the apparent absorption of light by reduced blood. The latter observations have led to the conclusion that a very considerable fraction of the light passing through a hemocyanin solution may be scattered by the hemocyanin molecules. The extent of this scattering determines in large part the color characteristic of the various bloods when examined either by reflected or transmitted light. By taking account of the amount of light scattered by the reduced solution, it has been found possible to determine the characteristic absorption spectrum of the molecular complex responsible for the bluish color developed when the hemocyanins combine with oxygen. In this way some information is obtained on the specificity of the oxygen-combining mechanism in the blood of different animals.

METHOD

Observations have been made upon the blood of the conch, *Busycon canaliculatum,* the horse-shoe crab, *Limulus polyphemus,* the squid, *Loligo pealei*, and the lobster, *Homarus americanus*. The bloods have been drawn by methods previously described (Redfield, Coolidge and Hurd, 1926), and preserved in the cold with toluene until prepared for observation. Under these conditions they may be kept with little change for many days. The bloods have been diluted to concentrations appropriate for the methods involved with sea water, distilled water, or various salt solutions, after which they have been allowed to stand overnight in the ice box in order to permit equilibrium with the modified environment to be reached. The material has then been filtered and placed in specially constructed tonometers in which it could be brought into equilibrium with various mixtures of gases. Each tonometer consisted of a cylindrical pyrex glass bottle of 200 cc. capacity, to the bottom of which a T-tube was sealed. The ends of the T were ground parallel to one another and were closed with optically flat glass plates sealed in position with DeKhotinsky cement. A chamber was thus provided, having an inside diameter of approximately one centimeter and a length which was in most cases exactly 3.3 centimeters. Following equilibration with the gas mixture, the sample of solution could be run down into the T-tube and the intensity of the light transmitted through it, measured. The specimens were oxygenated by filling the tonometer with oxygen or, in those cases where the character of the oxygen dissociation curve permitted, with air. Solutions containing reduced hemocyanin were prepared by evacuating the bottles after the introduction of the solution and refilling them with hydrogen. The bottles were then rotated for 15 minutes, after which the bottles were re-evacuated and again filled with hydrogen and equilibrated for an additional period of 25 minutes. The precision of the measurements is affected if the solutions are not perfectly clear. For this reason, the greatest care is necessary in filtering the solutions and in being sure that the dissolved materials are in equilibrium with their environment before filtration occurs, as otherwise small amounts of precipitated material may appear in the solutions before the photometric measurements are made. Reduced hemocyanin solutions are particularly troublesome because small amounts of material become insoluble during the mechanical disturbances incidental to evacuation and equilibration of the solutions. Under favorable circumstances, the insoluble particles produced in this way settle out if the specimens are allowed to stand for an hour or more prior to making the measurements. Under other circumstances the solutions remain slightly cloudy and the precision of the measurements is seriously interfered with. The second difficulty is in obtaining complete reduction of the solutions. Reduction appears to be satisfactorily attained by the method outlined above in the case of the bloods. In solutions of purified hemocyanin, because of the change in the shape and position of the oxygen dissociation curve, complete reduction is much more difficult to obtain. Further repetition of the processes of evacuation and equilibration with hydrogen would undoubtedly achieve the desired effect, but unfortunately such repetition increases the amount of insoluble material formed in such solutions and thus defeats its purpose. The use of chemical reducing agents has not been employed as those which have been tried have led to progressive changes in the color of the reduced material, which again defeats the objects of the experiments.

Measurements of the absorption of light by these solutions have been made with a König-Martens spectrophotometer constructed by Schmidt and Haensch. The light source of the instrument was illuminated by a Mazda projection bulb, the intensity of whose light could be controlled by a rheostat. The width of the slits was kept at 0.2 millimeters except at wave lengths less than 480 mμ, when it was increased to 0.4 or 0.6 millimeters as required in order to secure sufficient illumination. The calibration of the wave length scale of the instrument was checked from time to time and was found at all times to be accurate within 1 mμ. The precision of the instrument was also checked by the determination of the absorption of two colored glass filters, which had been standardized by the U. S. Bureau of Standards.

The absorption of light is indicated by the following equation:

$$\frac{I_0}{I} = \frac{\tan^2 a_0}{\tan^2 a_1}, \tag{1}$$

where I_0 is the intensity of incident light, I the intensity of transmitted light, a_1 the angle of the analyzing prism at which the fields match when a tube containing the solvent is placed in one of the beams of light; a_0 is this angle when the tube containing the solution is placed in this beam. In all cases, a_1 was determined with the absorption vessel filled with distilled water. Test showed that the result was the same, within the limits of observational error, in whichever beam the absorbing solutions were placed. In order to obtain results which might be compared with one another after the blood of different animals was examined, the results have been expressed in terms of the extinction coefficient, E, characteristic of each wave length as defined by

$$\frac{I}{I_0} = 10^{-Ed} \tag{2}$$

where d is the length in centimeters of the column of fluid. It follows that the extinction coefficient, E, is given by:

$$\frac{2(\log \tan a_0 - \log \tan a_1)}{d}. \tag{3}$$

In dealing with the absorption of light by hemocyanin, one is concerned particularly with the absorption of light by the complex formed when oxygen unites with hemocyanin. In this union it has been demonstrated that one atom of oxygen is combined for each atom of copper contained in the hemocyanin. The union appears to depend upon some grouping in the hemocyanin molecule, of which the copper forms an essential part. For convenience we will refer to this arrangement as the "chromatic group." For purposes of comparison it is interesting to determine the absorption of light in relation to the number of chromatic groups present. According to Beer's Law, the extinction coefficient of a substance in solution is proportional to its concentration. We have consequently expressed the absorption of light by the hemocyanin solutions in terms of E/c, where c is the concentration of copper in the solution expressed as milligram atoms per liter. An advantage of this notation also lies in the fact that the concentration of copper in serum may be readily obtained without the necessity of determining the number of grams of hemocyanin which are present, an investigation which cannot be made unless the hemocyanin of the species has been isolated and properly studied.

The Application of Beer's Law to Hemocyanin Solutions

The foregoing treatment assumes explicitly that in the absorption of light by hemocyanin solutions Beer's Law is valid and that in consequence E/c is a constant characteristic of the substance at each wave length. Quagliariello (1922) and Svedberg and Heyroth (1929) both present evidence that Beer's Law does not apply in the case of hemocyanin solutions. We have consequently examined this question care-

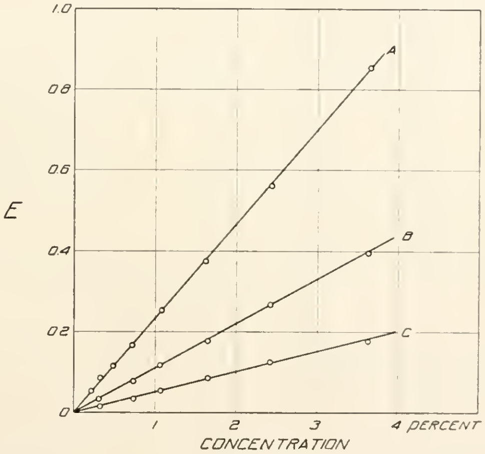

Fig. 1. Extinction coefficient of purified hemocyanin solutions of various concentrations.
A. Busycon canaliculatum at 570 mμ.
B. Limulus polyphemus at 580 mμ.
C. Limulus polyphemus at 480 mμ.

fully and have found no indication that Beer's Law is not valid when applied to such solutions and to such concentrations and at such wave lengths as we have employed. In Fig. 1 is shown the relation between the extinction coefficient of solutions of purified hemocyanin of two species made at various concentrations. In the case of *Busycon* and of *Limulus* the measurements were made at the wave length of maximal absorption and in the case of *Limulus* also at the wave length at which the absorption is minimal. In all three cases the relation between extinction coefficient and concentration is linear within the accuracy obtainable with photometric measurements on solutions of this character. Quagliariello's measurements were made upon native blood diluted with 2.5 per cent sodium chloride. It seemed possible that his anomolous results were due to alterations in the environment of the hemocyanin as the result of dilution, which might possibly affect the

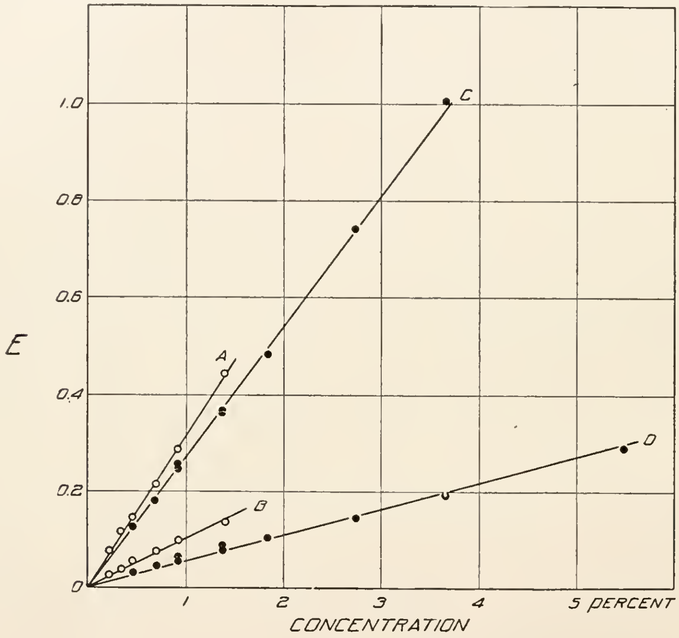

FIG. 2. Extinction coefficient of oxygenated and reduced solutions containing hemocyanin at various concentrations.

A. *Busycon canaliculatum* serum oxygenated. Dilution with 2.5 per cent NaCl. Wave length 570 mμ.

B. The same, reduced.

C. *Busycon canaliculatum* hemocyanin in potassium phosphate buffer solution oxygenated. Wave length 570 mμ. Dilution with phosphate buffer, 0.178 molecular phosphate; ionic strength 0.55; molecular fraction as K_2HPO_4 0.90.

D. The same, reduced.

degree of scattering of light by the protein, to be subsequently discussed. We have therefore made observations on the serum of *Busycon canaliculatum* similarly diluted with 2.5 per cent sodium chloride and have measured the extinction coefficient not only of the oxygenated but of the reduced solutions. The results are shown in Fig. 2, curves *A* and *B*. Again it appears that the relation between extinction coefficient and concentration is practically linear. As a further test we have made observations upon a solution of purified *Busycon canaliculatum* hemo-

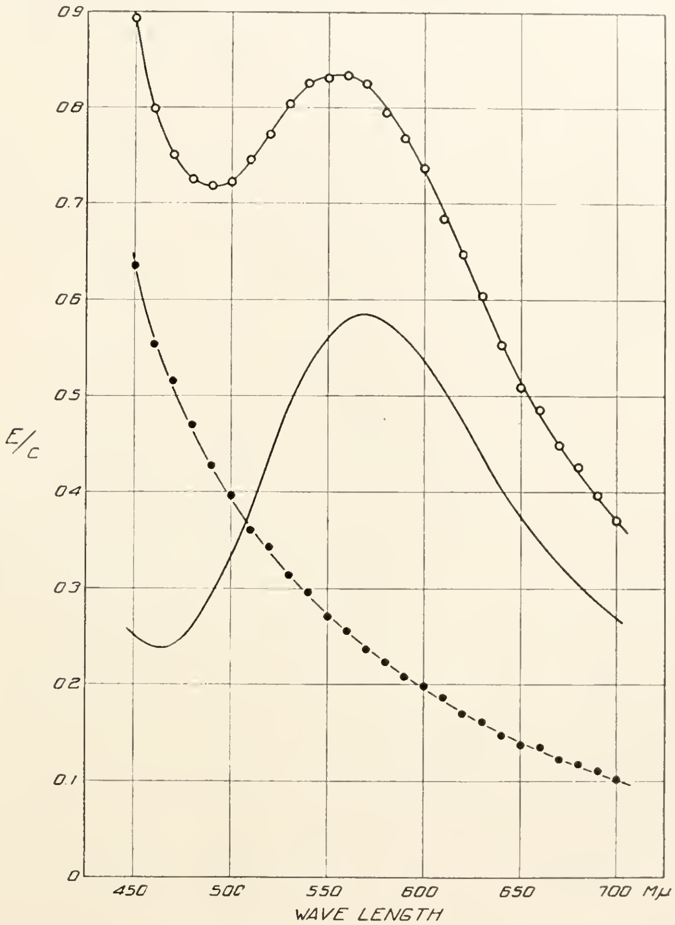

FIG. 3. Absorption spectra of blood of *Busycon canaliculatum*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.066 mgm. per cc.; dilution, 10 parts blood plus 18 parts H_2O plus 2 parts 0.1N NaOH; pH 9.6; length of absorption vessel 3.3 cm.

cyanin dissolved in potassium phosphate buffer and diluted carefully with a similarly buffered solution so as to maintain constant ionic strength. Measurements were made upon both the oxygenated and reduced solutions which again conform closely to the requirements of Beer's Law (Fig. 2, *C* and *D*). We consequently conclude that the assumption of Beer's Law is valid in connection with the observations discussed in this paper.

The Absorption Spectra of Native Blood

The typical spectra of the oxygenated and reduced bloods of *Limulus, Loligo, Busycon* and *Homarus* are presented in Figs. 3, 4, 5 and 6. Detailed descriptions of the solutions will be found in the

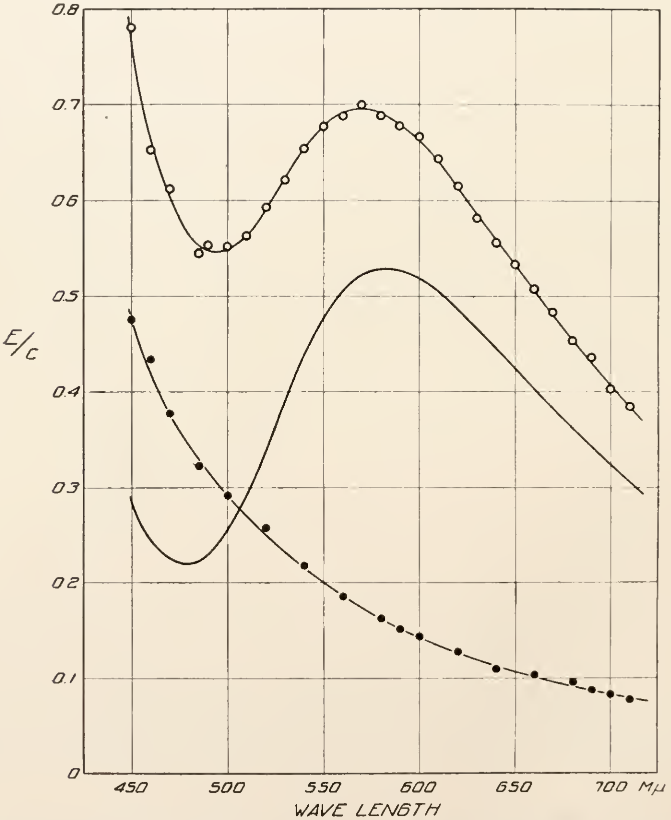

Fig. 4. Absorption spectra of blood of *Limulus polyphemus*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.081 mgm. per cc.; dilution, 20 parts blood plus 35 parts sea water plus 5 parts 0.08N HCl; pH 6.05; length of absorption vessel 3.3 cm.

legends of these figures. The upper curve in each case represents the absorption of light by the oxygenated blood, the lower curve by the reduced solution. A glance at the curves descriptive of the oxygenated blood serves to show a very considerable difference in the shape of each curve and in the general magnitude of the absorption. The curves do not differ markedly from those described by Quagliariello and others in the case of European forms belonging to related groups. The curves are alike in displaying a broad band of maximal absorption in the yellow with more or less increased transmission in the region of blue-green. It is in the relative values of the absorption in the blue-green and in the yellow regions that the curves differ characteristically, the species falling in the order *Busycon, Limulus, Loligo, Homarus* as the absorption in the blue-green region decreases. It is, of course, this difference which determines the observed colors of the different bloods.

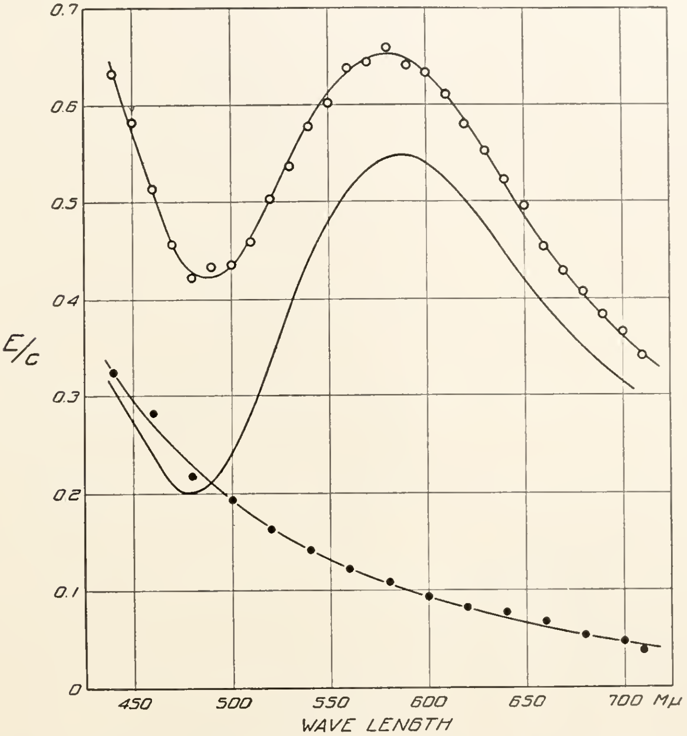

FIG. 5. Absorption spectrum of blood of *Loligo pealei*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.249 mgm. per cc.; dilution, one part blood plus 6 parts sea water; pH 8.11; length of absorption vessel 3.3 cm.

Spectra of Reduced Bloods

The spectra of the reduced bloods described by the lower curves in Figs. 3, 4, 5 and 6 deserve particular attention. It may be noted in each case, except that of the lobster, that these curves are similar in sweeping with gradual ascent uninterrupted by any obvious absorption bands as one passes from longer to shorter wave lengths. Comparing these curves for the different species, it may be noted that the absorption of light by the reduced blood is greatest in those forms in which the absorption by the oxygenated solution at the blue end of the spectrum is relatively high, the order being again *Busycon, Limulus, Loligo*. This fact may also be related to the observation of Redfield, Coolidge and Hurd (1926) that the Tyndall effect of the bloods studied by them decreases in the order *Busycon, Limulus, Loligo* and suggests that the absorption of light by reduced bloods may be due almost entirely to the scattering of light by the solution. The absence of

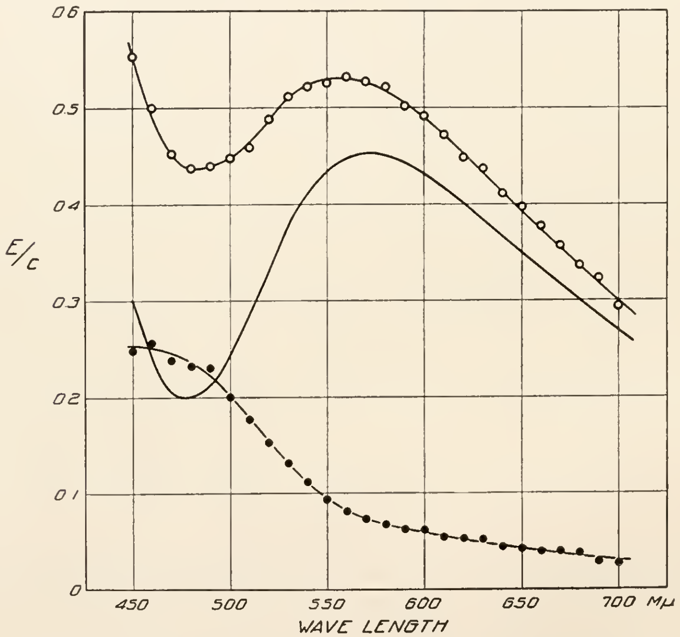

Fig. 6. Absorption spectrum of blood of *Homarus americanus* containing natural pigments. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.0505 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 7.87; length of absorption vessel 3.3 cm.

definite absorption bands in the reduced blood of these three species supports this hypothesis.

According to Lord Rayleigh (Strutt, 1871), when a beam of light passes through a medium containing particles small when compared with the wave length, the light of various wave lengths is scattered in proportion to the reciprocal of the fourth power of the wave length. The light, which is scattered at an angle of 90° from the incident beam, may be expected to be completely polarized provided the particles are spherical. Observation of the Tyndall beam emitted by hemocyanin solutions shows indeed that the Tyndall light is polarized, and inasmuch as the beam disappears entirely when viewed through a properly oriented Nicol prism, the polarization must be very nearly complete. Rayleigh deduces that the attenuation undergone by the beam as the result of

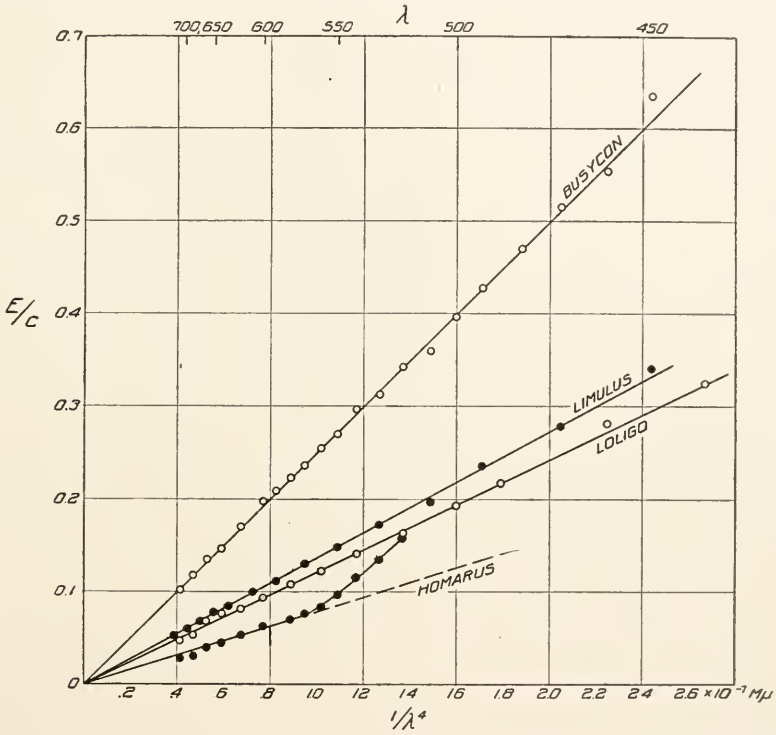

FIG. 7. Extinction coefficients, E/c, of reduced blood plotted against the reciprocal of the fourth power of the wave length, $1/\lambda^4$. For data regarding *Busycon*, *Loligo* and *Homarus* see legends to Figs. 3, 5 and 6. The data for *Limulus* is presented under Fig. 11 at pH 8.77. Concentrations, c, are expressed as milligram atoms of copper per liter.

scattering can be expressed by the equation

$$I = I_0 e^{-K\lambda^{-4}x}, \tag{4}$$

where x is the thickness of the scattering medium, λ is the wave length, and K is a constant characteristic of the solution in question. The validity of this equation was demonstrated in the case of mastic solutions by Abney and Festing (1886). Mecklenburg (1915) has shown that solutions of colloidal sulfur scatter light in proportion to the reciprocal of the fourth power of the wave length when the diameter of the particles falls between 5 and 93 mμ. For larger particles the relation no longer holds. The radius of the molecules of hemocyanin of *Helix* and *Limulus,* according to Svedberg and Heyroth (1929), are of the order of 10^{-6} centimeters or 10 mμ, so that we may expect the Rayleigh equation to apply in their case. From inspection of equations 2 and 4, it is obvious that for any given solution E or E/c should be proportional to $1/\lambda^4$. We may consequently test the hypothesis that the apparent absorption of light by bloods containing reduced hemocyanin is due to the scattering of light by the hemocyanin molecules by determining whether E/c at each wave length is proportional to the reciprocal of the fourth power of the wave length. In Fig. 7 the values of E/c for the various reduced bloods are plotted against $1/\lambda^4$. The lines so formed in the case of *Busycon, Limulus* and *Loligo* are straight lines which on exterpolation converge toward and meet at the origin, indicating that the Rayleigh formula does in effect describe the phenomena observed. It may be concluded consequently that the apparent absorption of light by the reduced blood of *Busycon, Limulus* and *Loligo* is to be attributed to the scattering of light by the dissolved hemocyanin.

The Corrected Spectra of the Chromatic Groups

The absorption of light by oxygenated blood must now be attributed to at least two components: the apparent absorption due to scattering and the true absorption due to the chromatic group. If these are the only factors involved, and if it be assumed that the scattering of light by the hemocyanin molecule is unaltered by the process of oxygenation, it is possible to correct the absorption spectra of the oxygenated bloods for the apparent absorption due to scattering and obtain a corrected spectrum of the chromatic group itself. If the attenuation undergone by the beam of light as the result of scattering is given by

$$\frac{I_1}{I_0} = 10^{-E_r d},$$

where I_1 is the intensity of "unscattered" light which would emerge were no other factors involved, and E_r is the extinction coefficient characteristic of the reduced material; and the further attenuation due to absorption by the chromatic groups is indicated by

$$\frac{I_2}{I_1} = 10^{-E_x d}$$

where I_2 is the final intensity of the emerged beam and E_x is the extinction coefficient expressing the effect of the chromatic group, then

$$\frac{I_2}{I_0} = 10^{-(E_x+E_r)d}.$$

The total absorption of light, however, is given by

$$\frac{I_2}{I_0} = 10^{-E_0 d}$$

where E_0 is the extinction coefficient of the oxygenated solution. Consequently,

$$E_0 = E_x + E_r.$$

The extinction coefficient of the chromatic group at unit concentration is consequently obtained by subtracting the value of E/c for the reduced solution from the value of E/c for the oxygenated solution at each wave length. This has been done, and the results are indicated by the intermediate curves in Figs. 3, 4, 5 and 6.

THE SPECTRA OF BLOOD CONTAINING OTHER PIGMENTS

The blood of the lobster requires special consideration because in addition to hemocyanin, this blood, in common with that of other crustaceans, contains the pigment tetronerythrin described by Halliburton (1885). Consequently the reduced blood of this species usually has a pinkish color and the bluish hue of the oxygenated blood has a more neutral color than that of the other forms if the pigment is present in sufficient amounts. As the result of the presence of this pigment, the spectrum of reduced lobster blood does not conform to the Rayleigh equation, as the lower curve in Fig. 7 shows. The tetronerythrin may be extracted from the blood by shaking the blood with chloroform. In Fig. 8 the absorption spectrum of the pigment extracted with chloroform is illustrated, the absorption of the dissolved pigment being compared with the absorption when the vessel is filled with chloroform. This substance possesses a maximal absorption at a wave length of 490 mμ and transmits nearly all of the incident light at wave lengths greater than 600 mμ. The apparent absorption of light due to scattering by the reduced blood of the lobster may consequently be arrived at approximately. By considering the absorption spectrum of the reduced blood at wave lengths greater than 600 mμ, it may be observed from Fig. 7 that these points fall along a straight line drawn from the origin of the diagram. Extending this line beyond 600 mμ indicates the degree of apparent absorption due to scattering at these wave lengths.

The presence of tetronerythrin or similar pigments, the color of which is unaffected by the oxygenation of the blood, does not interfere

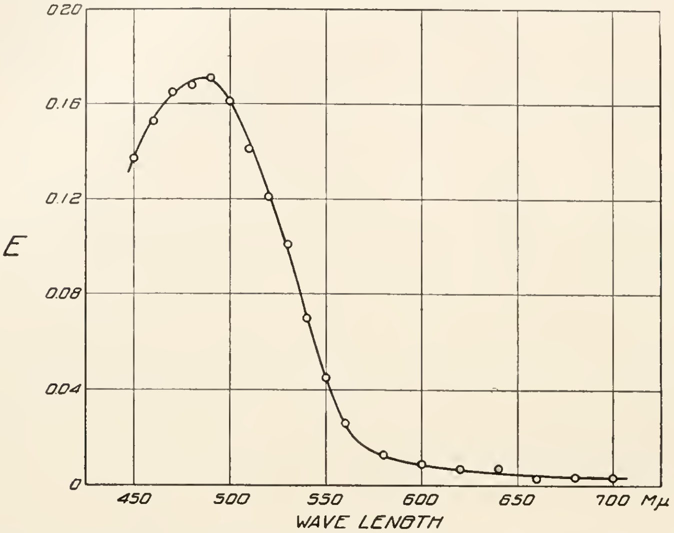

Fig. 8. Absorption spectrum of solution of the pigment extracted from lobster blood with chloroform. Concentration unknown; length of absorption vessel 3.3 cm.

with the determination of the corrected spectrum of the chromatic group. This may be demonstrated by examining the spectrum of blood from which the tetronerythrin has been extracted by chloroform. The spectra of oxygenated and reduced lobster blood so treated are illustrated in Fig. 9. It may be observed that the spectrum of the reduced solution no longer shows the irregularity due to the pigment. The corrected spectrum of the chromatic group may be seen to be almost identical with that obtained from the normal serum illustrated in Fig. 6.

A Comparison of the Spectra of the Chromatic Groups of Different Hemocyanins

It is a question of considerable interest to what extent the various respiratory proteins may be regarded as distinct "inventions of Nature," especially in that it is desirable to know whether the possession of similar or identical respiratory pigments indicates a generic relation between the groups of organisms possessing them. Recently much evidence has accumulated establishing the fact that the various hemocyanins are specifically different substances. This evidence consists in the demonstration of distinctive differences in the physical and chemical properties of these proteins. On the other hand, the evidence regarding the ratio between oxygen-combining power and copper content of the hemocyanins indicates that these substances have certain points in common, at least with regard to the portion of the molecule concerned with this function. To this complex when combined with oxygen we have applied the designation "chromatic group." A comparison of the spectra of the chromatic groups of different forms should consequently give evidence regarding the similarity of the chromatic groups in the

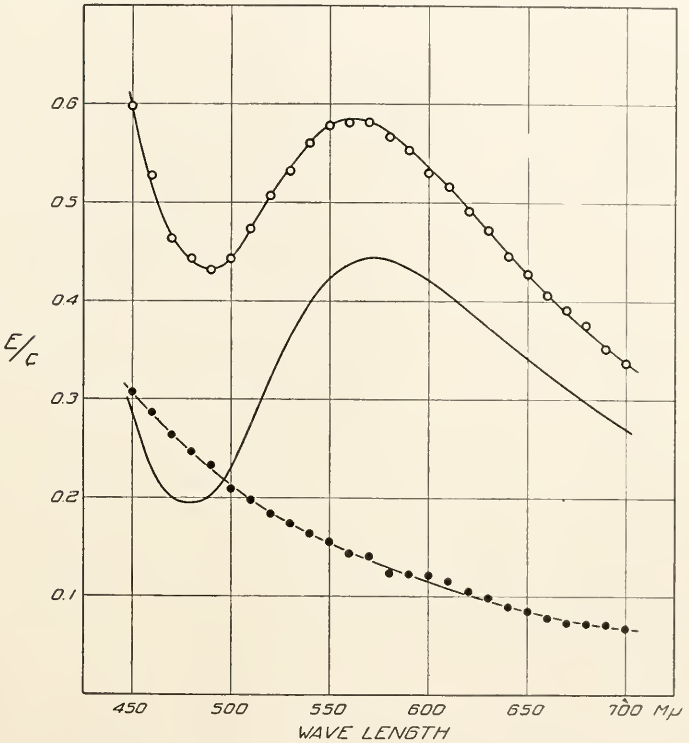

FIG. 9. Absorption spectrum of blood of *Homarus americanus* after extracting the pigment with chloroform. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, the spectrum of the chromatic group. Copper content of blood 0.0522 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 8.05; length of absorption vessel 3.3 cm.

hemocyanins of different classes of animals. In Fig. 10 the corrected spectra of the chromatic groups of the four species which we have studied are collected. It may be seen that on the whole the curves are strikingly alike, not only with regard to their shape, but also in relation to the actual quantity of light absorbed by equal numbers of chromatic groups. One is forced to the conclusion that the complexes responsible for these spectra are very much alike in each case. On the other hand, there are unquestionable differences between the spectra in the different cases.

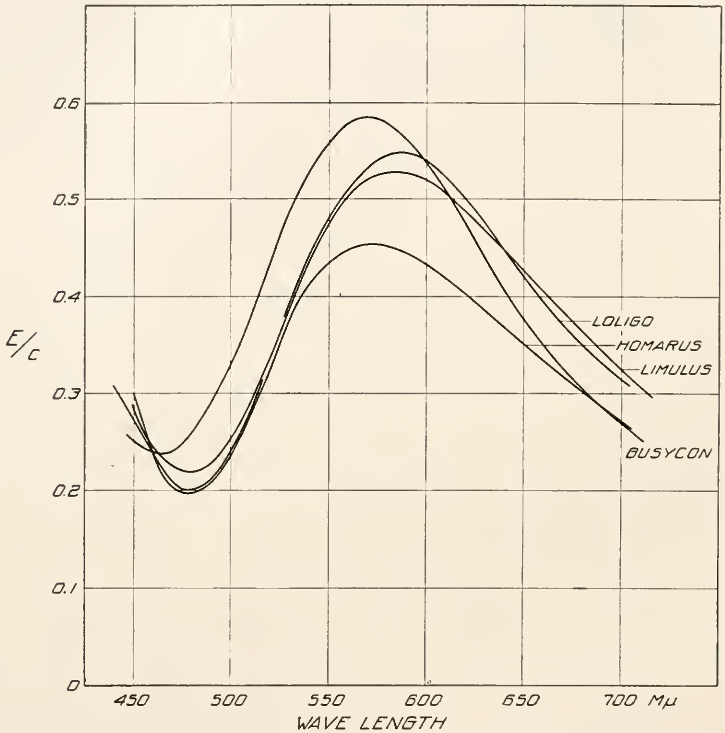

Fig. 10. Absorption spectra of chromatic groups of blood of *Busycon, Limulus, Loligo* and *Homarus*. For data see Figs. 3, 4, 5 and 6.

Factors Affecting the Absorption of Light by the Chromatic Group

A comparison of the chromatic groups of different species raises the question as to whether the differences observed may be attributed to differences in the chemical make-up of the body fluids in question.

It is consequently desirable to examine the effect of the nature of the solvent upon the absorption of light by hemocyanin solutions.

Hydrogen Ion Concentration.—The first point to be considered is the influence of hydrogen ion concentration upon absorption and scattering. When specimens of *Limulus* blood, to which various amounts of acid or alkali have been added, are examined, it is obvious to the eye that the color of the solution more alkaline than about pH 9 is different from the others. This difference is evident not only in the

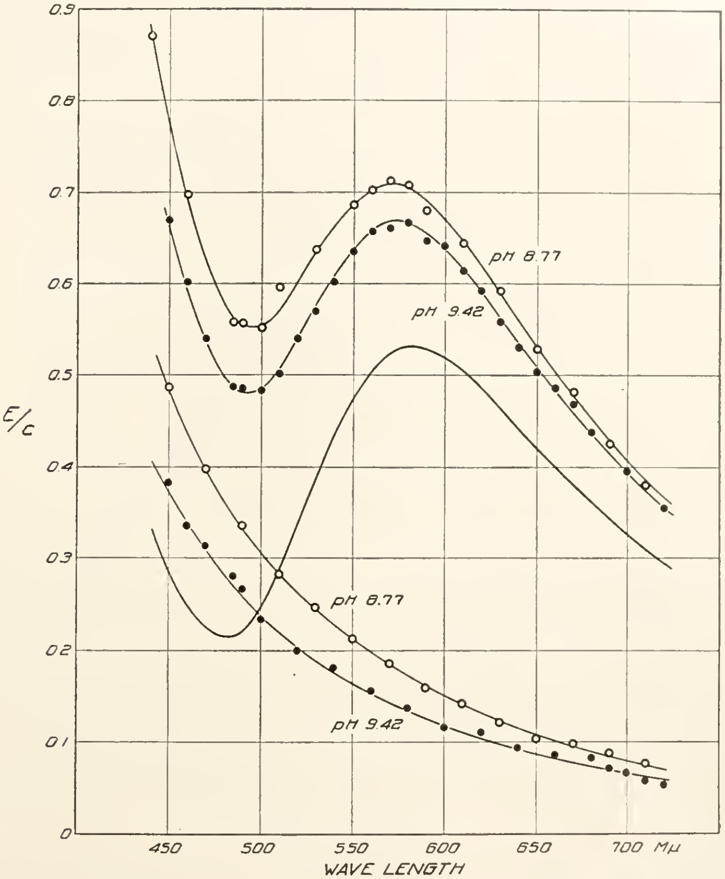

FIG. 11. Absorption spectra of blood of *Limulus polyphemus* at different hydrogen ion concentrations. Upper curves, oxygenated blood at pH 8.77 (hollow circles) and pH 9.42 (dots); lower curves, the same after reduction; intermediate curve, the spectrum of chromatic group, which is identical in both cases. Copper content 0.081 mgm. per cc.; length of absorption vessel 3.3 cm. Dilution which gave pH 8.77: 20 parts blood, 35 parts sea water, 5 parts 0.04N NaOH; dilution which gave pH 9.42: 20 parts blood, 35 parts sea water, 5 parts 0.1N NaOH.

oxygenated, but also in the reduced solutions, the oxygenated solution being a purer blue beyond pH 9 and the reduced solution having a fainter yellow color. In Fig. 11 are illustrated absorption spectra of specimens of oxygenated and reduced *Limulus* blood which were diluted with sea water, to which small quantities of sodium hydroxide had been added so that the solutions were at pH 8.77 and 9.44 respectively. With these curves the data presented in Fig. 4 should be compared, as the latter was obtained from the same blood brought to pH 6.05 by the addition of sea water containing small quantities of hydrochloric acid. The spectra illustrated in Figs. 4 and 11 account for the observed differences in color. The more alkaline specimen absorbs less light than the others in both the oxygenated and the reduced conditions.

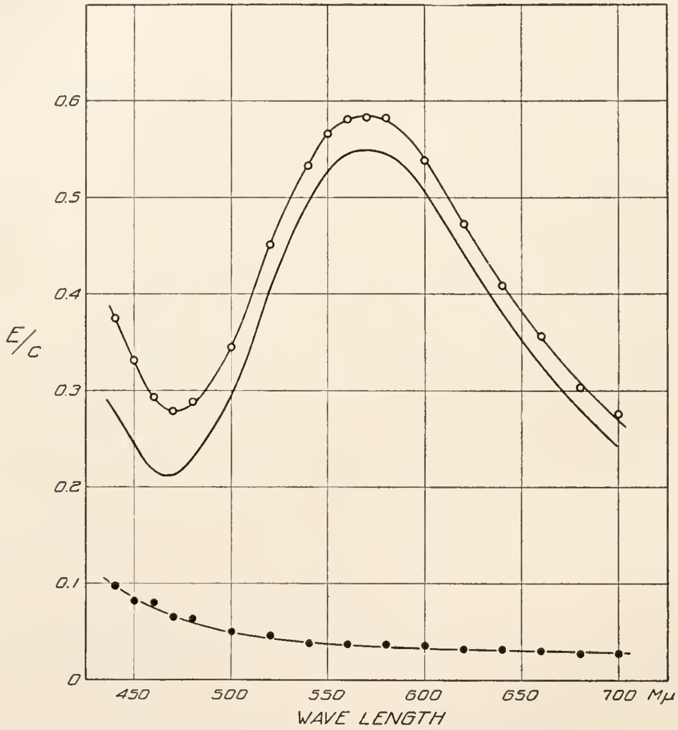

FIG. 12. Spectra of hemocyanin of *Busycon canaliculatum.* Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulfate followed by dialysis. It contained 0.129 grams hemocyanin per cc. and 0.308 mgm. Cu per cc. Dilution, 2 parts hemocyanin solution plus 14 parts H_2O plus one part 0.1N NaOH; pH 9.16; length of absorption vessel 3.3 cm.

It is clear also that the more alkaline solution scatters less light than do the others. Comparison of the corrected spectra of the chromatic groups shows, on the other hand, that the true absorption of light is not changed to a detectable degree by alterations in the hydrogen ion concentration. The differences in the spectra of the oxygenated bloods are sufficiently accounted for by the differences in scattering.

Salts.—A more profound alteration in the solvent may be obtained by purifying the hemocyanin so that it may be dissolved in water practically free of salts or other substances. By this means it is possible to compare the spectra of the chromatic groups of the different hemocyanins in solutions which are more or less identical. When solutions of pure hemocyanin are compared, it may be observed that the Tyndall phenomenon has undergone great diminution. Dilute solutions of reduced hemocyanin are practically colorless. The oxygenated solutions are of a purer blue color than when these substances are dissolved in the blood. These characteristics are all accounted for by an examination of the absorption spectra of the solutions, in which it may be observed that the reduced solutions appear to absorb very little light

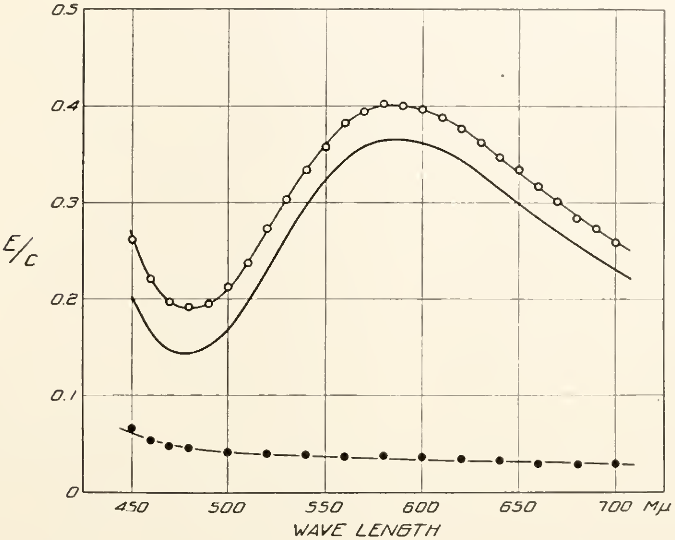

Fig. 13. Spectra of hemocyanin of *Limulus polyphemus.* Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulphate followed by dialysis. It contained 0.109 grams hemocyanin per cc. and 0.184 mgm. Cu per cc. Dilution, 5 parts hemocyanin solution plus 12.5 parts H_2O plus 2.5 parts 0.1N NaOH; pH 9.10; length of absorption vessel 3.3 cm.

and to absorb only slightly more light at the violet end of the spectrum than at the red end. Similarly the transmission of light in the blue-green region of the spectrum of the purified oxygenated hemocyanin is much greater than in the case of blood, and the absorption spectrum of the oxygenated solutions does not differ greatly from those of the corrected spectra of the chromatic groups. Spectra of purified hemocyanin solutions of *Busycon, Limulus* and *Homarus* are illustrated in Figs. 12, 13 and 14, together with the corrected spectra of the chromatic groups.

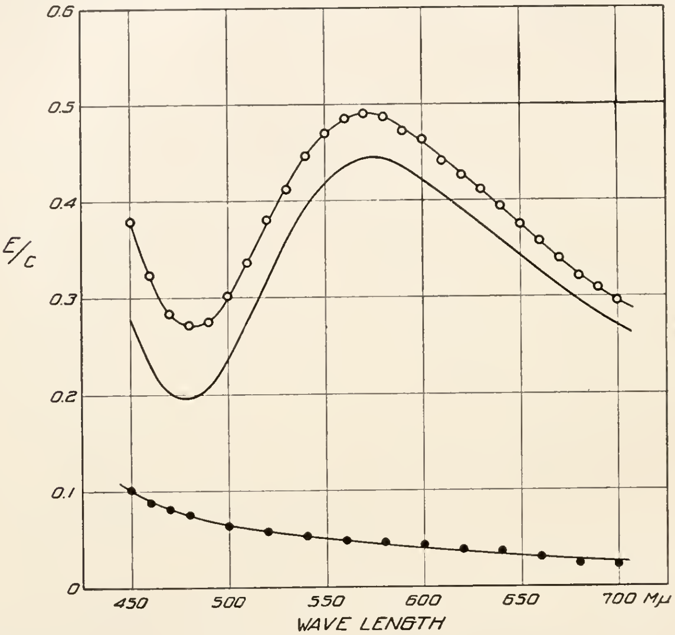

FIG. 14. Spectra of hemocyanin of *Homarus americanus*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin, purified by dialysis. Solution contained 0.1185 grams dry solids per cc. and 0.196 mgm. Cu per cc. Dilution: one part hemocyanin solution plus 3 parts H_2O; pH 8.10; length of absorption vessel 3.3 cm.

Comparison may now be made between the spectra of the chromatic groups of the purified hemocyanin and of the native blood. This is done in the case of these three species in Tables I, II and III. For accurate comparison the value of E/c for each wave length in the case of the purified hemocyanin is compared with its value in the case of the native blood. If the spectra of the chromatic groups are identical, this ratio should be the same at all wave lengths and have the value 1.0.

Examination of the tables showed that the ratio is not quite constant in each case at different wave lengths. The divergences are not large, but appear to be reproducible and indicate that the spectrum of the chromatic groups undergoes certain small changes as the result of the process of purification. The ratio also deviates from the value of 1.0 in each case. With *Busycon* and *Homarus* the purified material absorbs only slightly less light at each wave length than does a like concentration of hemocyanin in native blood. In the case of *Limulus* the discrepancy is much greater, amounting to about 30 per cent. These differences might be due to an alteration in the absorption of light by each chromatic group. On the other hand, they might be adequately accounted for on the assumption that as the result of the process of purification a certain quantity of the hemocyanin has lost the ability to combine with oxygen, which is necessary in order that the chromatic group be formed. The difference in the case of *Limulus* is sufficiently large to allow this possibility to be tested by a determination of the oxygen-combining power of the solution. The hemocyanin solution employed in this case contained 1.93 milligram atoms of copper per liter and might be expected to have an oxygen capacity of 1.93 milligram atoms of oxygen per liter. Actual analyses of the oxygen content of this solution when equilibrated with air yielded the values, 1.94, 1.95, 1.90 (mean 1.93) milligram atoms of oxygen per liter. Allowing 0.50 milligram atoms of oxygen per liter dissolved in the solution, one obtains 1.43 milligram

TABLE I

Comparison of absorption of light by chromatic groups of blood and purified hemocyanin of Busycon canaliculatum.

Wave Length	Hemocyanin	Blood	Ratio
mμ	*E/c*	*E/c*	
460	0.216	0.238	0.908
480	0.230	0.260	0.885
500	0.296	0.333	0.890
520	0.406	0.436	0.932
540	0.497	0.530	0.938
560	0.546	0.580	0.942
580	0.546	0.575	0.950
600	0.506	0.537	0.942
620	0.442	0.473	0.935
640	0.380	0.403	0.942
660	0.325	0.348	0.935
680	0.280	0.304	0.923
700	0.242	0.268	0.904

atoms as the actual oxygen-combining capacity. This value is 74 per cent of the theoretical, indicating that 26 per cent of the hemocyanin had lost its ability to combine with oxygen. The absorption of light by this solution is approximately 70 per cent of the absorption to be expected from the observations on hemocyanin as it occurs in native blood as Table II shows. It seems clear that in the case of this specimen at least, the discrepancy between the spectrum of blood and of the purified hemocyanin solution is due in large part to the modification of a portion of the hemocyanin in the process of preparation or preservation. The hemocyanin from which this specimen was prepared had been preserved for many months precipitated in half-saturated ammonium sulfate prior to preparation, and unfortunately we have not had an opportunity of re-examining this question with freshly collected hemocyanin.

TABLE II

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Limulus polyphemus.

Wave Length	Hemocyanin	Blood	Ratio
mμ	*E/c*	*E/c*	
460	0.165	0.244	0.677
480	0.144	0.220	0.655
500	0.168	0.257	0.655
520	0.231	0.338	0.683
540	0.298	0.438	0.680
560	0.345	0.506	0.683
580	0.365	0.528	0.692
600	0.362	0.518	0.699
620	0.344	0.486	0.708
640	0.314	0.446	0.705
660	0.284	0.403	0.705
680	0.256	0.362	0.708
700	0.231	0.324	0.714

These results lead to the conclusion that the observed differences in the extinction coefficients of hemocyanin in blood and in purified solutions may be accounted for largely by the denaturation of the hemocyanin in the process of preparation. They do not demonstrate that some difference in the absorption of light by the chromatic groups does not occur. Unfortunately the precision of the available methods for measuring oxygen capacity in these solutions is so low that changes cannot be detected unless they are relatively large. It may be concluded, however, that the spectra of the chromatic groups vary very little as the result of freeing the solutions from electrolytes and other impurities.

A comparison of the absorption of light by the reduced solutions of purified hemocyanin illustrated in Figs. 12, 13 and 14, with the curves for the absorption of light by the reduced serum of the corresponding species, shows that in the purified preparations, the scattering of light is much less than in the native blood. In the case of the lobster, the values of E/c characteristic of each wave length are, in the purified serum, about one-half those characteristic of the reduced blood. In the blood of *Busycon* and *Limulus,* the scattering of light is many times greater than in the purified preparations.

Table III

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Homarus americanus.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.232	0.240	0.968
480	0.196	0.202	0.972
500	0.237	0.244	0.972
520	0.317	0.332	0.956
540	0.393	0.410	0.959
560	0.435	0.447	0.975
580	0.444	0.451	0.984
600	0.421	0.432	0.976
620	0.391	0.402	0.973
640	0.358	0.366	0.979
660	0.327	0.333	0.984
680	0.295	0.300	0.984
700	0.270	0.268	1.007

The effect of purification upon the scattering of light may be shown to be due primarily to the removal of electrolytes from the solvent of the hemocyanin. By adding salt to purified hemocyanin solutions, the scattering effect is greatly increased. At the same time, the spectrum of the oxygenated solution approaches more nearly that of native blood. The spectrum of the chromatic group, however, appears to remain unchanged. These facts are illustrated by the data in Table IV, in which the values of E/c for oxygenated and reduced solutions of *Busycon* hemocyanin are compared when it is dissolved in water and when it is dissolved in a solution of potassium phosphate of an ionic strength approximately equal to that of native blood.

It may be concluded from the foregoing that the spectrum of the chromatic group is a relatively constant characteristic of hemocyanin solutions, influenced little if at all by the composition of the solvent provided that this does not interfere with the oxygenation of the material. On the other hand, the apparent absorption of light due to scattering varies greatly with the nature of the solvent and particularly with its salt content and hydrogen ion concentration. These facts are essential to the use of photometric methods in examining these solutions. They demonstrate that the measure of the absorption of light by the chromatic group may be a reliable index of the concentration of oxyhemocyanin. They also make it clear that in such measurements every precaution must be taken to control and take account of the degree of absorption due to the scattering of light.

TABLE IV

Absorption of light by hemocyanin of Busycon canaliculatum dissolved in potassium phosphate buffer; phosphate concentration, 0.357 molar; pH, 7.7.

Wave Length	Oxygenated in Phosphate	Reduced in Phosphate	Chromatic Group in Phosphate	Chromatic Group—Salt-Free	Ratio
mμ	*E/c*	*E/c*	*E/c*	*E/c*	
460	0.559	0.345	0.214	0.216	1.009
480	0.514	0.288	0.226	0.230	1.017
500	0.544	0.237	0.307	0.296	0.964
520	0.625	0.208	0.417	0.406	0.974
540	0.691	0.178	0.513	0.497	0.969
560	0.722	0.131	0.567	0.546	0.963
580	0.695	0.132	0.563	0.546	0.971
600	0.632	0.118	0.514	0.506	0.985
620	0.560	0.105	0.455	0.442	0.972
640	0.477	0.092	0.385	0.380	0.987
660	0.415	0.078	0.337	0.325	0.965
680	0.361	0.071	0.290	0.280	0.966
700	0.324	0.069	0.255	0.242	0.950

In a preliminary report on the present investigation (Redfield, 1929) it was suggested that the relative size of the particles of hemocyanin could be deduced from the scattering of light with the aid of the Rayleigh theory. However, Raman (1927) has developed a theory of scattering by colloidal solutions, in accordance with which it appears possible to relate the observed optical phenomena to the osmotic pressure of the solutions. The experiments of Loeb on gelatin indicate that the variations in osmotic pressure of protein solutions induced by altering the nature of the solvent, which he accounted for by the considerations involved in Donnan membrane equilibria, are in the necessary direction and have sufficient magnitude to account for the observed variations of scattering in terms of Raman's theory. Until this possibility is examined critically, it is improper to draw inferences concerning the degree of aggregation of hemocyanin in blood from the phenomenon of scattering.

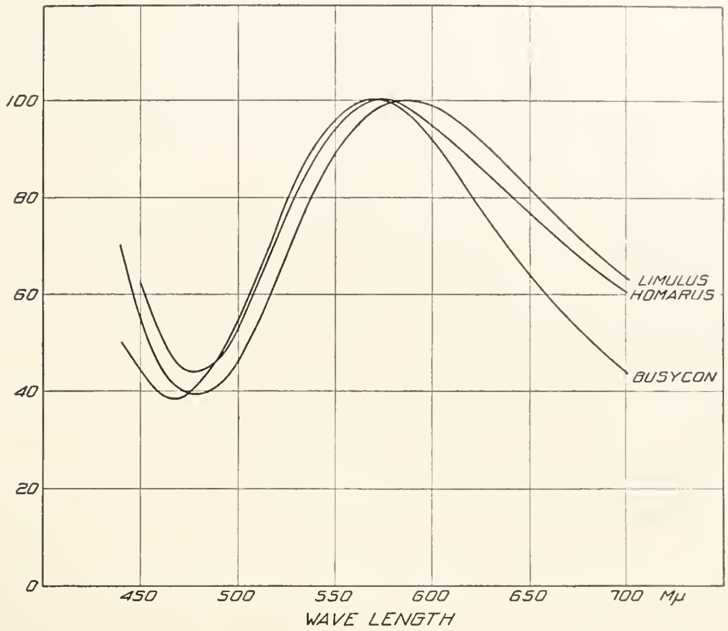

Fig. 15. Absorption spectra of chromatic groups of purified hemocyanins of *Busycon, Limulus,* and *Homarus.* The ordinate is an arbitrary scale such that the value of E/c for each spectrum is 100 at the wave length of maximal absorption. For data see Figs. 12, 13 and 14.

Comparison of the Chromatic Groups of Purified Hemocyanin in Aqueous Solutions

In order to compare the spectra of the chromatic groups of the different purified hemocyanins it is necessary to employ some method which disregards the errors due to the denaturation of a certain portion of the hemocyanin in the process of purification, as the foregoing discussion indicates that data may not give us accurate information with regard to the concentrations of oxygenated hemocyanin in the various preparations. The spectra of the chromatic groups of the different hemocyanins described by Figs. 12, 13 and 14 have consequently been reduced to an arbitrary scale in which the maximal intensity of absorption in the yellow region has been taken as 100. The data so obtained are plotted in Fig. 15. Comparing these curves, it is evident that even in aqueous solutions the spectra of the chromatic groups are markedly different. One may conclude consequently that the characteristics of these spectra are not dependent upon the chemical peculiarities of the body fluids of the different animals but on specific differences in the chromatic groups themselves or on the influence of the specific characteristics of the hemocyanin molecule as a whole upon that portion which is concerned with the transport of oxygen.

Summary

1. The absorption of light by the blood and by purified preparations of the hemocyanin of the conch, *Busycon canaliculatum,* the horse-shoe crab, *Limulus polyphemus,* the squid, *Loligo pealei,* and the lobster, *Homarus americanus,* has been studied. It is shown that the absorption of light by solutions containing oxygenated hemocyanin may be resolved into two components: (a) that due to the true absorption by the chromatic group formed by the union of oxygen with the portion of the molecule containing copper and (b) that due to the scattering of light by the dissolved protein.

2. In the analysis of the spectrum of the blood of the lobster, the absorption of light by the pigment tetronerythrin has been taken into account.

3. The spectrum of the chromatic group of a given species varies very little, if at all, as the result of alterations in the hydrogen ion concentration and salt content of the solution.

4. The spectra of the chromatic groups of the different species display a considerable similarity, indicating a close chemical relationship. There exist, however, definite differences in the spectra of each species which persist after the process of purification and indicate definite specific differences in the various hemocyanins.

5. The scattering of light varies widely among the different species and is responsible in large part for the difference in appearance of the bloods, particularly when viewed by reflected light. The scattering is modified greatly by changes in the composition of the solution, being diminished in the more alkaline solutions and particularly in solutions free from electrolytes.

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ANOPLOPHRYA MARYLANDENSIS N.SP., A CILIATE FROM THE INTESTINE OF EARTHWORMS OF THE FAMILY LUMBRICIDÆ

CECILE CONKLIN

(From the Department of Biology, Goucher College and the Department of Protozoology, School of Hygiene and Public Health, Johns Hopkins University)

Material and Methods

Anoplophrya marylandensis, a new species of astomatous ciliate, was discovered in the intestine of *Lumbricus terrestris* (Linn., 1758) and *Helodrilus caliginosus* (Savigny, 1826). Many of the infected hosts were immature forms, making identification uncertain. Assistance in identifying the earthworms given by Dr. Frank Smith, formerly of the University of Illinois, is gratefully acknowledged.

The hosts infected with this form were obtained from a limited area in the city of Baltimore. Hosts of the same species obtained from three other localities within the city failed to show this form. Those from two of the other regions showed no intestinal ciliates. In the infected area the incidence of infection was 29.13 per cent.

The parasites were usually numerous in the infected worms. They were found only in the anterior third of the intestine, and were most numerous just in back of the gizzard. The organisms were obtained after anesthetizing the host with chloretone. The body of the worm was slit along the mid-dorsal line exposing the intestine into which short incisions were made in different regions. The contents of the intestine were removed from these regions with a tooth-pick and smears were made in physiological salt solution. Smears were fixed with Schaudinn's fixative and were stained with Heidenhain's iron hæmatoxylin. Parasites were studied in the living condition by placing the contents of the intestine into a watch glass of physiological salt solution.

Description

This new species was uniformly ciliated and flattened. The body was extremely thin and did not appear to be more than one-fifteenth its width in thickness. Stained specimens were not found in such a position that thickness could be measured. The dorsal and ventral surfaces of specimens just removed from the intestine appeared to be perfectly flat. Some of them became slightly rounded after they had been in the physiological salt solution for a few minutes. The change in form was evidently due to a difference in osmotic pressure. The body was rounded at the posterior end and slightly pointed at the anterior end; it was broader at the anterior end than at the posterior. The greatest breadth was just anterior to the center of the body.

One hundred specimens were measured with an ocular micrometer at a magnification of 1000. The following dimensions and biometrical data were entered as being typical.

Breadth in microns	Length in microns										
	36	40	44	48	52	56	60	64	68	72	
16		1	1								2
20	1	5		3							9
24	1	2	5	5	9	1	2	1			26
28		1	7	2	1	2	1	3	2		19
32	1		1	2	7	8	5	3			27
36					1	4	5	3			13
40								1	1	2	4
	3	9	14	12	18	15	13	11	3	2	100

	Length	*Breadth*
Range	36 to 72 μ	16 to 42 μ
Mean	52.7 ± 0.6 μ	28.6 ± 0.4 μ
Standard Deviation	8.6 ± 0.4 μ	5.6 ± 0.3 μ
Coefficient of Variation	16.4 ± 1.0%	19.8 ± 1.0%

The body was covered with long cilia arranged in longitudinal rows which were close together. The number of rows varied from 31 to 40 in five specimens. These longitudinal rows of cilia converged slightly at each end of the parasite. The cilia of twelve specimens averaged 7.3 μ in length. The average length of these twelve parasites was 50.8 μ. In general the length of the cilia varied directly with the size of the parasite, the larger parasites having the longer cilia.

There was a thin pellicle which covered a transparent layer of ectosarc. The ectosarc was confined to a thin layer except at the anterior end where it made up the greater part of the pointed region. The endosarc was very granular when stained with Heidenhain's iron hæmotoxylin and it appeared to have many chromatin granules scattered throughout.

There were two nuclei, a large ribbon-like macronucleus and a small spherical micronucleus. The macronucleus extended through the long axis of the body and was nearly as long as the body itself. In 25 specimens the average length of the macronucleus was 43.1 μ while the average length of these same parasites was 55.5 μ. The average width of the macronucleus was 5.9 μ while the average width of the parasites was 32.7 μ. The outline of the macronucleus was very irregular and several showed fine projections extending into the endosarc. (Fig. D.) Often a clear space appeared around the macronucleus in stained specimens. This was probably due to shrinkage.

The micronucleus was very small and appeared to be spherical in specimens not undergoing division. It was surrounded by a layer of clear protoplasm, which may also have been due to shrinkage, for the micronucleus could only be seen in stained specimens. The micronucleus was found about mid-way between the anterior and posterior ends of the body and was lateral to the macronucleus. It was always found on the side of macronucleus opposite to that of the row of contractile vacuoles.

If the parasites were numerous in the host, about every tenth one was in the act of dividing. Division was transverse. Figs. B, C, D, and E show different stages in transverse fission. The micronucleus underwent a mitotic division with the formation of a spindle and chromosomes. There were apparently only a few chromosomes but their extremely small size made them very difficult to count. Fig. C shows four chromosomes that have just divided and the two groups are separating from each other. In the prophase of division the chromatin granules appeared to be lined up in two strings with successive enlargements which made them look like two strings of beads.

Tarnogradsky (1914) and Cépède (1909) have described posterior budding in other species, but in the present study no specimens in unequal division have been found nor did any have other individuals attached to them.

No conjugating individuals were seen as has been described by Collin (1909) in *A. brasili* Leger and Duboscq.

There was a single longitudinal row of contractile vacuoles along one side of the macronucleus. The number varied from two to five. The vacuoles in even a single specimen were of widely different sizes. As many as five different specimens have been watched for one half hour at a time and never have any of the vacuoles been seen to contract. The specimens have been placed in a suspension of India ink but no expulsion of fluid from the vesicles was seen. Lankester (1870) described the contraction of vacuoles in an Opalina which has since been classified as *Anaplophrya naidos* by Kent (1880) and more recently as *Bütschliella naidos* by Mackinnon and Adam (1924). Lankester said the contractile vacuoles in this form contracted very suddenly, slowly reappearing in the same place. He found that the collapse occurred a little less frequently than twice a minute. Tarnagradsky (1914) found that the period of contraction of the contractile vacuoles in *A. inermis* Stein was from 1.5 to 15 minutes. Cépède (1910) found that the contractile vacuoles of *A. alluri* failed to contract if the animals were removed from their normal habitat.

Behavior

Parasites could not be kept alive more than twenty-four hours outside the host. Physiological salt solution and various dilutions of it with distilled water to 0.25, 0.5, 0.375, 0.625, and 0.75 of its normal strength were used. The parasites lived longest in the normal solution. Those in the less concentrated solutions soon developed large blisters on the body and died.

The parasite turned on its long axis as it swam, frequently making complete turns but often making only a half turn from a horizontal position to a horizontal position and back again. It was observed to make this half turn even when the liquid in which it was confined was sufficiently deep so that the animal was not cramped nor was it prevented from making a complete turn. When it did make a complete turn it was in a clockwise direction.

The parasite swam rapidly and underwent no deformation due to the mechanical action of movement, though the body seemed flexible enough to bend when it struck an obstruction.

Differential Diagnosis

As far as available literature goes to show, *Anoplophrya marylandensis* n.sp.,[1] can with one exception be distinguished from all other species in the genus which fall within its size range (36 to 72 μ in length) by the fact that it has one row of contractile vacuoles instead of two rows. The exception is *A. parva* Rossolimo, from an aquatic oligochæte and is separated from *A. marylandensis* by the position of the micronucleus. In *A. marylandensis,* the micronucleus occurs upon the side of the macronucleus opposite the contractile vacuoles; in *A. parva,* both micronucleus and contractile vacuoles are on the same side

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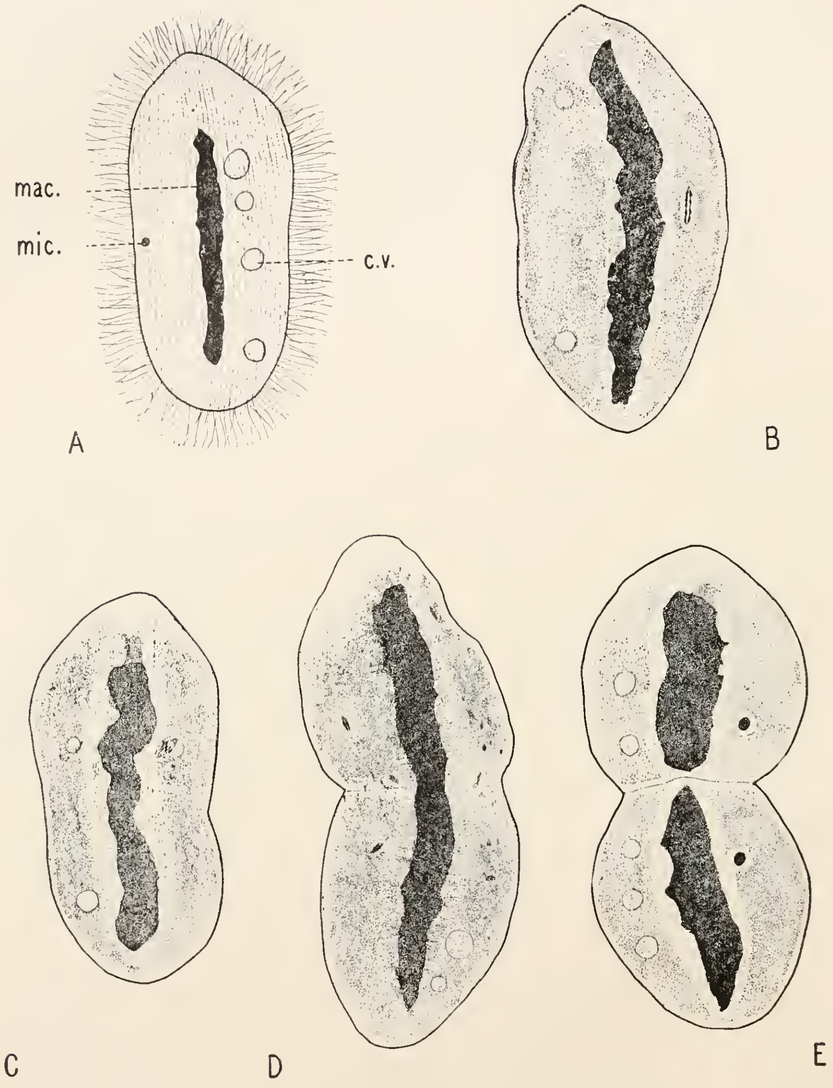
[1] A type specimen of this species has been deposited in the National Museum at Washington, D. C.



Explanation of Figures

All specimens stained with Heidenhain's iron hæmotoxylin. All drawings made with camera lucida at a magnification of × 750.

Fig. *A*. *Anoplophrya marylandensis* n.sp., normal resting individual. Mac., macronucleus; mic., micronucleus; c.v., contractile vacuole.

Figs. *B*, *C*, *D*, *E* represent successive stages in division of *A. marylandensis*. *B*, prophase, *C*, metaphase, *D* and *E*, telophase.

of the macronucleus. Furthermore, *A. marylandensis* is much broader (averaging about 28 μ in breadth) in comparison to *A. parva* (breadth, 17 μ).

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THE GROWTH OF LARVÆ OF *AMBYSTOMA MACULATUM* UNDER NATURAL CONDITIONS

W. T. DEMPSTER

(From the Zoölogical Laboratory of the University of Michigan)

Introduction

Attempts to describe the increment in length and weight during the larval history of amphibians are confronted with either of two difficulties. If the animals are raised in the laboratory at a constant temperature, the normal or optimum food conditions cannot be duplicated readily; if animals are collected periodically from their natural habitat, the conditions of life there are so variable as to produce data difficult to describe. Accordingly the literature is neither extensive nor consistent.

Davenport (1897, 1899) presented a few data upon the weight increment of larvæ of " the common frog " under laboratory conditions. Three stages of growth are recognized; first a period of slow growth accompanied by abundant cell division, then a period of rapid growth due to imbibed water, and finally a period of equally rapid growth in which the increment is due to increase both in organic substance and water.

Schaper (1902) has provided data still more complete on the larval growth of *Rana esculenta* under laboratory conditions. The daily growth in weight and volume of these tadpoles is slight at first, gradually becoming greater and attaining a maximum value at about eighty days. During the following week these values fall to about half as the animal undergoes metamorphosis. During the first fourteen days of development the organic matter and ash remain constant (13 mgm. and 1 mgm. respectively). Weight increment during this period is due solely to imbibition of water. The percentage of solid then increases until maximum size is attained; during metamorphosis this percentage is further increased. Schaper's data on length increase cannot be easily interpreted, probably because too few specimens were considered.

Robertson (1923) apparently unaware of Schaper's work has attempted with indifferent success to convert Davenport's data into a mathematical expression. He believed that the weight increment in the frog tadpole could be expressed by a single symmetrical sigmoid curve.

Studies on salamanders have shown variable rates of growth. When the length-age data of Eycleshymer and Wilson (1910) on *Necturus* under laboratory conditions and those of Bishop (1926) dealing with the animal under natural conditions are plotted it may be seen that during the first three (or four) months of development (except for a period before the embryonic axis is straight) the length increases at a constant rate.[1] The nearly uniform yearly growth to the period of sexual maturity, which Bishop records, suggests that aside from thermal variations the rate of linear growth may be constant from year to year.

Wilder (1924), on the other hand, has shown that under natural conditions the rate of linear growth varies at different times during the larval history in *Spelerpes bislineata.* Although she disregarded the embryonic development, subsequent growth stages are recorded. The post-embryonic stage, until the yolk is absorbed, is the period of most rapid growth. The typical larval period during the fall and winter of the first year is a time of slow growth. The latter part of this period during the spring of the second year is characterized by rapid growth. The premetamorphic stage during the fall and winter of the second year involves a period of slow growth, then a period of fluctuating growth. The metamorphic stage in the summer of the third year is a period in which the catabolic changes are more pronounced than the anabolic.

It must be noted that *Spelerpes* does not become terrestrial until it has spent two years of its life in an aquatic habitat; *Necturus* is permanently aquatic. It seems likely that a more typical method of growth would be found in salamanders which have an aquatic stage lasting for only a single season. The increase in length of *Ambystoma* has been studied by two observers. Uhlenhuth (1919), who has studied *A. opacum* under constant conditions, stated that the rate of growth seems to be proportional to the velocity of metamorphosis (rate of growth × age at metamorphosis = constant). He does not, however, describe the growth rate of various periods of development.

Patch (1927) has described the length increase in three groups of *Ambystoma* as consisting of two sigmoid curves, one embryonic and the other larval. The point of junction of these two curves is 15.61 mm. in *A. maculatum,* 14.07 mm. in *A. tigrinum,* and 11.96 mm. in the axylotl.

In order for curves of length increase to represent a fundamental phase of growth, the relation between weight and length for successive stages must be constant, as Miss Patch has assumed. In view, however, of the marked changes in body form during the embryonic life of *Ambystoma* it seems unlikely that the " indices of build " are uniform. It is necessary to have data on both length and weight, at least to the point in development where the body form assumes nearly constant larval proportions, in order to correctly appreciate the body increment.

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[1] Actually, Bishop's data shows a slight variation from the constant growth rate over a period of two weeks in July. The climatological records of the U. S. Weather Bureau for the Saegertown, Pa. region give a rise in temperature during this period which undoubtedly accounts for the fluctuation.

MATERIAL AND METHODS

During the spring of 1928, the author located a salamander pond sufficiently well populated with spawning *Ambystoma maculatum* to indicate that eggs and larval specimens could be obtained throughout the season. The present study involving about 1700 specimens is the outcome of two years of systematic collection from that habitat.

Delhi Pond is a shallow, sheltered, leafy-bottomed forest pond in the environs of Ann Arbor. It has a maximum area of one-twelfth acre and a maximum depth of about four feet; it is ordinarily a permanent pond but became dry during the season of 1929. In addition to *A. maculatum,* the usual invertebrate fauna, *A. tigrinum* and *Rana cantabrigensis* were present.[2]

From the time when eggs were first observed until the salamanders metamorphosed, periodic random collections were made, the intervals between successive collections being seldom more than a week. Thirty to forty specimens formed a sample although occasional collections, particularly during the early stages, amounted to more than a hundred specimens. Specimens were collected by dredging the bottom of the pond with a hand net formed of a yard of wire netting stretched between two poles. All parts of the pond were sampled so that the collection is quite representative. The maximum-minimum temperatures of the pond were recorded at the time of collection.

The specimens were brought to the laboratory alive, anesthetized and measured, mutilated specimens having been discarded. During the non-motile stages anesthesia was not required. The eggs and embryos up to the period of hatching were placed for measurement upon the stage of a binocular microscope fitted with a camera lucida.

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[2] Although B. G. Smith (1911) stated that *Ambystoma tigrinum* and *A. maculatum* are not found in the same habitat in the Ann Arbor region, the author found both salamanders in abundance in the present location. The two populations are more or less independent of one another and it is not evident that the larger salamander through its predatory activities seriously affects the *A. maculatum* population. The eggs and larvæ of the two species could not be confused easily since the egg clutches, the time of hatching, the size and appearance of embryos and larvæ are distinctly different.

The image of the embryo could thus be superimposed on a properly calibrated scale. The larger, later specimens were measured by means of drafting calipers and millimeter rule. In the blastula and non-motile stages the maximum diameter or length irrespective of the curvature of the body axis in relation to the yolk mass was recorded. The length taken in later stages when the axis was linear was the maximum length.

The average weight at different developmental stages was also determined: The anesthetized salamanders were placed in a tared crucible and weighed after the excess water had been absorbed by pipette and filter paper. They were then dehydrated for several days in a drying oven at 95–97° C. and the dry weight determined. Following this the sample was incinerated over a Meeker burner for two to ten hours and the ash weighed.

RESULTS

Changes in Weight to the Period of Metamorphosis.—During the year 1928, the first eggs were found on April 3. On August 18, many specimens had begun to metamorphose at a weight of about 1200 mgm. A week later there were relatively few specimens in the pond. During the year 1929 the first eggs were collected on March 27, and on August 14, a number of specimens, at approximately 800 mgm. had metamorphosed. When the average weight of a sample of salamander eggs, embryos or larvæ is plotted against the age, as in Figs. 1 and 2, the rate of growth may be expressed as a curve. The weight increment was slow at first, gradually increasing to the middle of June when the rate of increase became more and more rapid. By the middle of July the rate of growth was at its maximum. In the first week of August the growth rate was markedly reduced. Finally growth became negligible and metamorphosis occurred. The weight increase may be thus described as a single sigmoid curve. Under natural conditions the first stages of growth were considerably prolonged, due to the low water temperatures of spring. During the larval and premetamorphic stages the temperatures are more nearly the same. The terminal period of growth is very brief, so short in fact, that the curve of *Ambystoma* increment shows a marked variation from the curves of autocatalytic growth in other animals. During both years the same general type of sigmoid curve is demonstrated, although the actual weight values are considerably different.

Linear Increase to the Time of Metamorphosis.—The curve formed by plotting length against age shows a slight deviation during the first four or five weeks of development. From this point on the curve is sigmoid. Growth increment is gradually increased to a period within three to four weeks of metamorphosis when the rate of increase is considerably lowered, and finally becomes negligible. The deviation during the early stages of development, which Miss Patch has interpreted as a distinct period of growth, involving the typical sigmoid growth rates, is due to the form changes of the embryo. The embryonic axis from the neurula to the early limb bud stages is curved around the yolk mass. Increase in length of this axis during the early growth stages does not result in equivalent increase in the total length of the embryo. It is not until the embryonic axis becomes straightened that the total length shows marked increase.

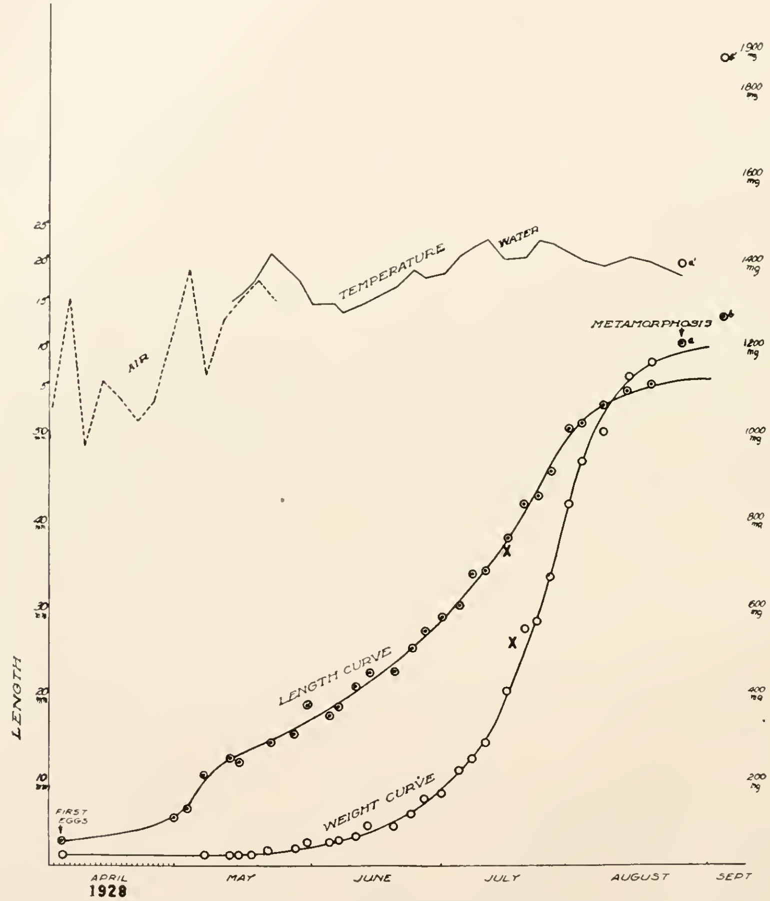

Fig. 1. A curve showing the relation between average length and weight of larval specimens of *A. maculatum* at various ages. Data of 1928.

That the departure from a single sigmoid curve during the early development is not a distinct phase of growth may be demonstrated. When an " index of build " (Length ^{3}/Weight) is computed (Table I), it is clearly indicated that length and weight are not directly associated during the early stages. This index varies constantly to a period shortly before hatching when the embryonic axis becomes linear. It is fairly constant, however, for the free living larval stages.

A group of experiments carried under approximately constant temperature conditions gives indication that the degree of curvature may vary under environmental circumstances. Four groups of salamanders at the neurula stage were placed at approximately constant temperatures of 4° C. ± 1, 13° C. ± 1, 19° C. ± 1 and 27° C. ± 1. When the four groups of length-age data acquired from these animals were plotted in such a way as to rule out the change in growth rate due to temperature, that is, when all the data, after allowance is made for appropriate thermal coefficients of growth, are plotted as though the animals were raised at 19° C., the shape of the curve is not the same for each group. The linear increase in the 4° sample was practically

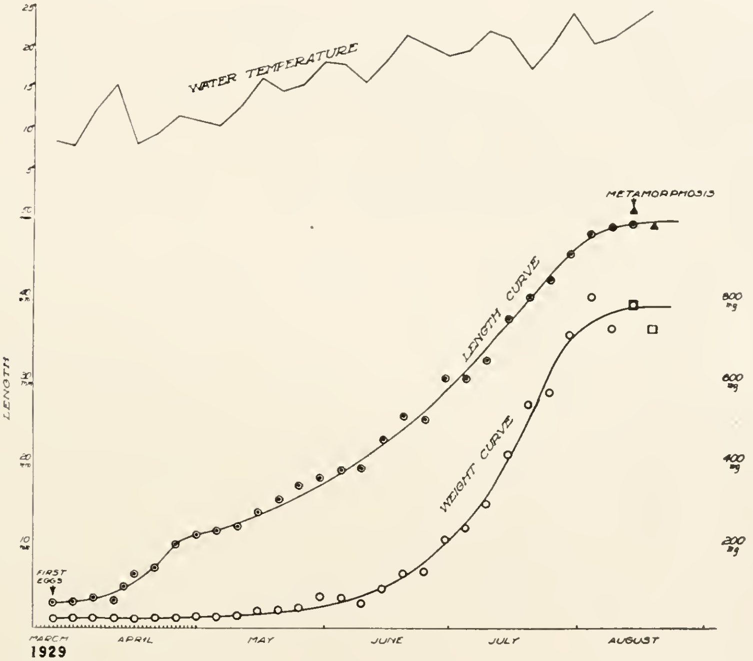

FIG. 2. Curve of growth showing weight and linear increment. Data of 1929.

TABLE I

Giving the average lengths, weights and analyses at different stages of embryo and larval A. maculatum. Data of 1929

Date	Mean Length	Average Wet Weight	$\frac{\text{Length}^3}{\text{Weight}}$	Water	Dry Weight	Ash	Water	Dry Weight	Ash	Stage
	mm.	*mgm.*		*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*	*per cent*	
Mar. 27.....	2.21 ± .08	6.16	1.8							7–9 (Harrison)
Apr. 1.....	2.37 ± .11	7.32	1.8	4.98	2.34	.097	68.03	31.97	1.32	10–13
Apr. 6.....	2.75 ± .31	6.89	3.0	4.73	2.17	.070	68.58	31.42	1.01	15–20
Apr. 11.....	4.55 ± .99	8.43	11.2	5.70	2.73	.081	67.62	32.38	.96	26–31
Apr. 16.....	5.81 ± .14	6.98	28.1	4.51	2.48	.095	64.54	35.46	1.36	33±
Apr. 21.....	6.56 ± .15	7.85	35.9	5.53	2.33		70.38	29.62		36±
Apr. 26.....	9.45 ± .90	9.50	88.8	6.93	2.57		72.94	27.06		34–39
May 1.....	10.51 ± .77	9.78	118.6	7.54	2.24		77.08	22.92		39–41
May 6.....	11.09 ± .52	11.28	120.8	8.82	2.46		78.19	21.81		
May 11.....	11.76 ± .61	11.52	141.1	9.52	2.00	.20	82.63	17.37	1.74	42–43 Hatching
May 16.....	13.41 ± .61	25.3	95.2	22.87	2.43	.30	90.38	9.62	1.18	42–45
May 21.....	15.01 ± .75	27.34	123.6	25.89	2.45	.29	94.68	5.32	1.04	45 (yolk nearly gone)
May 26.....	16.75 ± 1.00	36.38	129.1	33.43	2.96	.38	91.86	8.14	1.04	46+ (eating?)
May 31.....	17.61 ± 1.33	63.64	85.8	58.66	4.98	.62	92.17	6.42	.97	
June 5.....	18.52 ± 1.64	58.42	108.7	53.47	4.95	.70	91.52	8.47	1.20	
June 10.....	18.65 ± 1.69	44.96	144.4	40.67	4.29	.45	90.45	9.54	1.00	
June 15.....	22.2 ± 2.33	83.56	130.8	75.68	7.88	.92	90.56	9.43	1.10	
June 20.....	25.18 ± 2.19	119.55	133.8			1.50			1.26	
June 25.....	24.7 ± 2.85	121.40	124.1	109.35	12.05	1.45	90.08	9.93	1.20	
June 30.....	29.93 ± 2.48	204.37	132.1	184.13	20.24	2.53	90.09	9.91	1.24	
July 5.....	29.8 ± 2.50	235.68	114.8	211.24	24.44	2.96	89.63	10.37	1.25	
July 10.....	32.0 ± 2.89	289.41	113.3	255.48	33.93	3.78	88.28	11.72	1.30	
July 15.....	37.21 ± 2.32	412.54	124.5	366.97	45.57	5.91	88.95	11.05	1.43	
July 20.....	39.78 ± 3.02	538.21	117.1	477.16	61.05	7.61	88.66	11.34	1.41	
July 25.....	41.94 ± 2.94	565.00	130.1	496.00	69.00	8.07	87.79	12.21	1.43	
July 30.....	45.10 ± 3.71	709.45	128.4	618.03	91.43	11.10	87.11	12.89	1.56	
Aug. 4.....	47.70 ± 4.17	798.68	135.8	694.13	104.55	11.55	86.91	13.09	1.45	
Aug. 9.....	48.50 ± 2.68	722.50	157.9	624.30	98.20	11.62	86.41	13.59	1.61	
Aug. 14.....	48.92 ± 2.36	785.63	148.8	673.77	111.86	13.30	85.76	14.24	1.69	
	50.50	784.50	164.1	667.25	117.25	15.25	85.05	14.95	1.94	Terrestrial
Aug. 19.....	48.70 ± 2.56	717.10	161.1	590.60	126.50	16.90	82.35	17.65	2.36	"

a straight line growth. Some deviation from this type of growth was found in the 13° data, more in the 19° data and still more deviation in the 27° data. It is very unlikely that the weight differs in these groups. High and low temperatures apparently affect the efficiency of the cardiovascular mechanism so that atypical individuals are eventually produced. Under high temperature conditions the embryo folds around the yolk, develops rapidly and eventually straightens its axis; under the low temperature yolk is not readily utilized and the head and tail buds from the time of their formation extend away from the yolk mass rather than lie close to it.

In all the data provided by salamander collections under natural conditions, it may be noted that the " probable error of length " has a more or less constant ratio to the average length determinations of the various stages. The population may thus be considered to be fairly homogeneous concerning the individual growth rates.

Relation of Weight and Length.—Aside from the deviation between length and weight in the early embryonic stage, due to embryonic foldings, there are certain other fluctuations. Occasional samples from two other ponds compared with 1928 curves indicated that for a certain weight, there were considerable variations in length. In the period before the animals began to feed there was little difference in these values but later the differences were marked. When the 1929 curves are superimposed on the 1928 curves this relation is brought out clearly. The curves of linear growth and the weight curves practically coincide to the point *X* of Fig. 1. From this point to the period of metamorphosis the variation is great. A higher average " index of build " is found for the data of the first year as compared with that of 1929.

Time of Metamorphosis.—It seems quite probable that the actual time of metamorphosis under natural conditions is associated with the conditions of life in the pond. In August, 1929, the pond under consideration became dry. The growth weight as evidenced by the curve showed a marked slowing down toward the end of July, while in the previous year, under more favorable conditions, this was not evident until the first week in August. During this first year, in fact, there were specimens in the pond for at least two weeks after most of the salamanders had metamorphosed.[3] This laggard group was formed of relatively large specimens (Fig. 1, *a*, *a'*, *b*, *b'*). It seems quite probable that these specimens had not yet entered the third period of growth, *i.e.* the terminal period of slow growth. During the second year, when the pond became dry, two records, one before the pond became dry and the other immediately afterward, are available on the length and weight of recently metamorphosed specimens. In the first of these both values are higher than in the second. The second group was undoubtedly " forced " by the drying of the pond to metamorphose before reaching the stage at which the first group metamorphosed.

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[3] R. G. Harrison (Correlation in the development and growth of the eye, etc. *Arch. f. Entw.-Mech.*, Bd. 120, 1929) has figured three curves for the post-embryonic linear growth of *A. maculatum* larvæ under laboratory conditions. These curves, which are sigmoid, indicate that the rate of growth is accelerated with increased feeding and, in contrast to the present data, that the length at metamorphosis is constant (47–50 mm.) under different feeding conditions. A similar curve is given by L. S. Stone (Heteroplastic transplantation of eyes between the larvæ of two species of *Amblystoma*. *Jour. Exp. Zoöl.*, Vol. 55, 1930).

Allee (1911), who has studied the seasonal succession of pond fauna, has indicated that there is a periodic change in numbers of species and individuals found in forest ponds. There is an increase in numbers of species which is slow during the spring months and rapid in early summer, less marked in July and in late August the number falls to the spring value. There seems to be a correlation between the period of highest productivity of the pond as reported by Allee and the period of rapid growth of the salamanders recorded here.

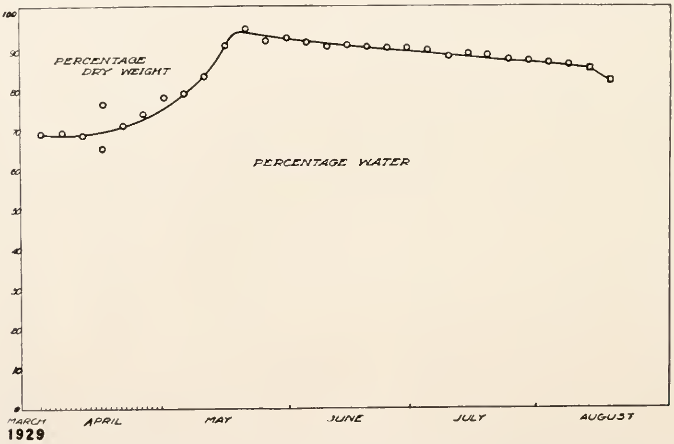

FIG. 3. Graph showing the relative percentage of water and organic substance in larval salamanders of different ages. Data of 1929.

Relations of Water, Solids and Ash to Growth.—The eggs shortly after they were laid had a weight of 7.32 mgm. consisting of 4.98 mgm. of water and 2.34 mgm. of solid, of which .097 mgm. was ash. During the embryonic period the dry weight was fairly constant. Actual increase was associated with increase in inorganic matter and water (Table I). The ash percentage, however, was practically constant while the water increased in this period from 68 to 94 per cent. After the animals began to eat, the dry weight increased considerably so that the percentage of water decreased. To the period of metamorphosis there was a gradual increase of inorganic matter from 1 to 2 per cent. Water per cent decreased from 94 to 85 per cent and the percentage dry weight increased from 6 per cent to 15 per cent. This relationship is brought out in Fig. 3. Until the animals began to feed, growth was purely a process of hydration; afterwards it was due both to imbibition of water and to increase in organic and inorganic materials. These findings are in accord with the work of Davenport and Schaper on the Anura. Recently metamorphosed specimens showed still further decrease in the percentage of water content. Data on the percentage of water in older metamorphosed specimens (Table II) show that this early decrease may be later compensated. The proportion of dry weight, ash and water, however, seems to be variable for the land forms. Two specimens from an indoor aquarium in December showed a decrease in water content to 80 per cent body weight and an increase in inorganic matter to 4 per cent.

TABLE II

Showing the relative content of water, solids and ash in terrestrial stages of A. maculatum.

LENGTH	WET WEIGHT	$\frac{\text{LENGTH}^3}{\text{WEIGHT}}$	WATER	DRY WEIGHT	ASH	WATER	DRY WEIGHT	ASH
mm.	*grams*		*grams*	*grams*	*grams*	*per cent*	*per cent*	*per cent*
September 1929								
50.5	.603	214	.491	.112	.011	81.51	18.49	1.77
71	1.659	218	1.412	.247	.031	85.12	14.88	1.86
76	2.504	177	2.206	.298	.032	88.10	11.90	1.27
82	4.616	120	4.063	.553	.084	88.02	11.98	1.82
88	4.709	146	4.012	.697	.101	85.10	14.80	2.14
93	5.602	144	4.955	.647	.102	88.45	11.55	1.82
104	5.875	193	4.939	.936	.124	84.07	15.93	2.11
December 1928								
136	9.432	269	7.848	1.584	.427	83.21	16.79	4.53
138	8.792	301	7.044	1.748	.366	80.12	19.88	4.16

SUMMARY

1. Growth in weight of embryonic and larval *Ambystoma maculatum* from the time that eggs are deposited to the period of metamorphosis may be expressed as a single sigmoid curve.
2. The length curve, except for a short period before hatching when the embryonic axis is curved, is likewise sigmoid.
3. The *Ambystoma* population of a pond is quite homogeneous, the specimens metamorphosing at approximately the same time.

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4. Under natural conditions the relation between weight and length from year to year seems to be constant during the stages before feeding. Later the relationships are variable because of feeding differences.

5. Growth to the time of food ingestion is associated with imbibition of water. Later growth to the time of emergence of the salamanders is correlated with a process in which the percentage of water content decreases. During this period the inorganic constituents gradually increase.

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THE EFFECTS OF TEMPERATURE CHANGES UPON THE CHROMATOPHORES OF CRUSTACEANS

DIETRICH C. SMITH[1]

(From the Harvard Biological Station, Soledad, Cienfuegos, Cuba and the Zoölogical Laboratory, Harvard University.)

Temperature changes as they affected the chromatophores of crustaceans were not neglected in the researches of early investigators, those of Jourdain (1878) being the first recorded in the literature to consider the possible influence of this factor. At 5°–6° C., according to his observations, the rapidity at which color changes occurred in *Nica edulis* was appreciably reduced, ceasing entirely as the temperature approached nearer to zero. At this point the animals were almost transparent, except for areas partly covered with matted white spots. Jourdain removed the eyes of those crustaceans and noted that the reddish color assumed at room temperatures, under such circumstances, disappeared entirely when the temperature of the water was lowered only to reappear again on the restoration of the temperature to its former level. Matzdorff (1883) observed no effect whatever of either high or low temperatures upon the chromatophores of *Idotea tricuspidata*. Somewhat later however, Gamble and Keeble (1900), after a few observations upon *Hippolyte varians*, reported observable color response following exposure to heat and cold. Their specimens in common with most other crustaceans possessed several differently colored pigments, reds and yellows predominating, located with one exception in discrete bodies or chromatophores. During the day the reds and yellows were usually expanded, but at night these pigments were retracted into their chromatophore centers and if it were not for a blue pigment, diffused at this time throughout the tissues and free from any chromatophore, the animals would be colorless. Gamble and Keeble selected three of these transparent blue prawns, which they called "nocturnes," and placed one in water at 15.5° C. (60° F.), one in water at 8° C. and the last in water at 32° C. (93° F.). The first animal, in reality the control as it was kept under normal temperature conditions, turned greenish-brown as was to be expected. The second one at 8° C. maintained the nocturnal blue color, showing after thirty-five minutes some traces of recovery, though one hour later this was still incomplete.

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[1] National Research Fellow in the Biological Sciences.

The prawn placed in 32° C. was almost immediately killed by the heat, but remained nevertheless a brilliant nocturne for several hours, even though during the first five minutes of this experiment the temperature descended to 28° C. (83° F.).

Menke (1911) experimenting with *Idotea,* produced a contraction of the chromatophores in about 15 minutes by raising the temperature of the water from 11.5° C. to 20.5° C. This contraction was sustained for about one hour when the pigment partially re-expanded. Five and one-half hours later on, lowering the temperature to 12° C., the chromatophores again became completely expanded. But if at this time, instead of lowering the temperature of the water, it was raised to 30° C., the chromatophores also re-expanded completely. Complete expansion was also produced by lowering the temperature from 14° C. to 4° C. Doflein (1910), working with *Leander xiphias* placed several specimens in complete darkness at 5°–8° C. for two to three days. At the lapse of this time the chromatophores and the tissues of the animal were completely impregnated with blue pigment, all other pigments being completely retracted into their respective centers. But as Fuchs (1914) points out, these results might follow either from continued exposure to cold or to darkness. Megušar (1911) working with *Gelasimus, Potamobius, Palæmonetes, and Palæmon,* observed an expansion of the chromatophores on the sudden transfer of any of these animals from water at 16°–18° C. to water at 25°–30° C. Similarly a contraction of the chromatophores followed a sudden transfer from water at 25°–30° C. to cooler water at 16°–18° C.

The results of these experiments are admittedly confusing, though as Fuchs (1914) observed, no reasonable doubt can be entertained as to the ability of temperature changes to produce an effect of some sort upon the pigmentary responses of the crustaceans. Further investigation of the subject was thought desirable in the hope of ascertaining, if possible, just how important a factor the action of heat and cold is in determining the distribution of the chromatophore pigment of this group. For this purpose a fresh water shrimp, kindly identified for me as *Macrobrachium acanthurus* Wiegmann by Dr. W. L. Schmitt of the United States National Museum, was selected as the subject of the experiments. These shrimps were obtainable in large numbers from the Arimao river and its immediate tributaries in the vicinity of the Harvard Biological Station, Cienfuegos, Cuba. I am happy to acknowledge my thanks and appreciation to Dr. Thomas Barbour for his assistance in putting the facilities of the Harvard Cuban Station at my disposal.

When caught, the chromatophore pigments of these shrimps were more or less extended, giving the animal a reddish-brown color. This color varied somewhat with the size of the animal, the smallest being the lightest. As collected, the shrimps ranged from 2 cm. to 10 cm. in length, measured from rostrum to telson. Males varying from 2 cm. to 3 cm. in length were selected for the experiments. Females were rejected, as at this time their abdomens were practically opaque owing to the fact that they were carrying their eggs.

A word or two regarding the color changes of *Macrobrachium* will be an aid to the understanding of what is to follow. Taken to the laboratory and placed in white glazed porcelain bowls, the shrimps in daylight soon became transparent and colorless; microscopical examination of the abdomen and telson showing the chromatophores to be completely contracted. If such animals were placed upon a black background, they assumed a dark reddish color with the chromatophores well expanded. A somewhat superficial examination disclosed the presence of two types of chromatophore pigments, both apparently located in the same chromatophore, one being reddish-brown in color and the other yellow. These facts were derived from a microscopical examination of the living pigmentary units. Detailed histological study of the chromatophores was not attempted.

Occasionally under somewhat varying conditions, animals were seen with an unmistakable bluish color observed both in the light and in the dark. The blue pigment producing this color when examined under the microscope was clearly not confined to the chromatophores, but was free in the tissues, though its concentration did appear greater about the processes of the pigmentary centers. Gamble and Keeble (1900) reported that a blue color was the regular accompaniment of the nocturnal phase of *Hippolyte varians,* a phase characterized by the retraction of all other pigments into their respective centers. According to their statement, the blue pigment in *Hippolyte* responsible for the nocturnal coloration arises as a discharge product of the chromatophores, leaving these organs on the contraction of the yellow and red pigments, and apparently being derived from them. Left free in the tissues, the blue pigment is permanently divorced from its point of origin and persists in coloring the body of the prawns until it eventually disappears.

In these experiments upon *Macrobrachium acanthurus* determinations were first made of the action of heat and cold upon the color changes in normal shrimps. The method used was as follows: Two or three animals were placed in white porcelain bowls and covered with water at room temperatures. To this was added either warm or cold water, as desired, until the particular temperature demanded by the experiment was reached. Here it was either kept constant or altered as necessary. The responses of the shrimps to temperature changes when kept upon a black background were tested in the same manner.

Numerous experiments with normal shrimps adapted to white backgrounds demonstrated conclusively that such animals darkened when exposed to temperatures either high enough or low enough to stimulate the chromatophores. Surprising as it may seem, once the response was complete, no criteria of any sort could be established separating the darkening produced by heat from that produced by cold. The color assumed in either circumstance was a deep red-brown, while microscopical examination showed the pigments of the chromatophores to be equally well extended at high and low temperatures. The protocols of the two following experiments may be taken as typical of many others:

2:13—28° C.	Two colorless shrimps previously kept on a white background for a day were placed in a white porcelain bowl and small pieces of ice added to the water.
2:16—10° C.	No change in color.
2:18—10° C.	Shrimps appear slightly reddish.
2:22—15° C.	Shrimps somewhat darker.
3:00—15° C.	Shrimps a pronounced brown.
3:25—28° C.	Shrimps still brown.
9:30—28° C.	Shrimps colorless.

2:13—28° C.	Two colorless shrimps previously kept on a white background for a day were placed in a white porcelain bowl and warm water gradually-added.
2:16—36° C.	No change in color.
2:18—36° C.	Shrimps faintly reddish.
2:21—36° C.	Shrimps pronouncedly brown.
3:00—28° C.	Shrimps colorless.

In all of the experiments the appearance of the red-brown color was more rapid at high temperatures than at low. With heat only 10–15 minutes were necessary to make the animal completely dark, while with cold 30–45 minutes were required. But regardless of whether the shrimps were exposed to heat or to cold, once the point of maximal darkening was reached, the intensity of the color was equal in both cases.

When the animals were subjected to warmth the lowest temperature capable of expanding the chromatophores was found to be 35° C., while temperatures as high as 40° C. could be withstood without subsequent death, though at this temperature and slightly below it, the shrimps remained motionless, and with the exception of gill movements showed no signs of life. Therefore, within the range of 35° C. to 40° C. the color of the shrimp is determined by the temperature of its environment rather than the type of background on which it happens to be. Similarly shrimps placed in water colder than 6° C. died immediately, while any temperature above 15° C. and, of course, below 35° C., failed to produce an expansion of the chromatophore pigment. Therefore, between 6° C. and 15° C. the color of the shrimp is also determined by temperature rather than background. It might be well to mention here that the temperature of the water in which the shrimps normally lived ranged from 25° C. to 30° C.

As a check upon these results and to determine whether there was any possibility of temperature changes producing a contraction of the chromatophore pigment, experiments similar to those just described were performed upon dark shrimps while they were upon a black background. But such animals when exposed to various temperatures ranging between 6° C. and 40° C. showed no alteration whatever in the expanded condition of their chromatophores.

Recovery of normal color and activity was the rule when shrimps subjected to effective temperatures were returned to water at about 28° C. But this recovery was more rapid in shrimps treated with warmth than those treated with cold. The former required but 30 to 40 minutes, and the latter 6 to 7 hours before normal temperatures and a white background again brought their chromatophore pigment to complete contraction.

Tests were also made of the responses of blinded shrimps to temperature changes, blinding being accomplished by cutting off the eyes at the base of the eye stalk. Shortly after this operation the pigment of the chromatophores began to expand and within an hour or so, regardless of background, this expansion was complete and the animals were red-brown in color. Shrimps in this condition placed in warm and cold water and left so for an appreciable length of time—two to three hours—showed no color change of any sort. Similarly shrimps anaesthetized with 0.05 per cent chloretone, failed to show color responses to either heat or cold. Neither high nor low temperatures are then capable of exerting any contracting effect upon the pigment of the chromatophores, even when these organs are removed from the influence of any stimuli directly or indirectly produced by the retina.

As Perkins (1928) has shown in *Palæmonetes*, the withdrawal of pigment into the centers of the crustacean chromatophore is controlled by a hormone elaborated in the eye stalks, a fact which was later substantiated by Koller (1928) on *Crangon* and *Leander*. Possibly then, as temperature changes acted to expand the chromatophore pigment, there was an inhibition by heat or cold of the mechanism controlling the production of this contracting secretion. Before such an hypothesis could be tested, it was necessary to ascertain definitely whether or not such a secretion played a part in governing the chromatic responses of *Macrobrachium*. Consequently Perkins' experiments were repeated upon this animal. Five or six shrimps were paled by placing them upon a white background for a day or more, after which their eyes were removed and thoroughly macerated in 2 cc. of 0.7 per cent NaCl. One tenth cc. of the resulting solution was then injected into the abdomens of several shrimps in the dark condition and with well expanded chromatophores. In all cases the following reactions were noted: Shortly after injection, 5–10 minutes, the shrimps began to assume a bluish color which gradually increased in intensity until within 30 minutes it had reached its maximum; this was followed by a gradual retraction of the pigment into the chromatophore centers, a retraction which persisted until the shrimps had assumed a transparent blue color. These results closely parallel the effects reported by Perkins in *Palæmonetes,* even to the formation of the blue color, and offer complete substantiation of his findings. As control experiments 0.1 cc. of the extract was injected into the abdomens of several shrimps in the light condition with no observable effect. Similarly injection of 0.1 cc. of 0.7 per cent NaCl into blinded shrimps produced no pigmentary response.

The existence of a hormone produced by the action of light upon the retina and released into the circulation to affect a contraction of the chromatophore pigment is then demonstrated in the shrimps used in these experiments. Is the formation of this hormone in any way inhibited by either high or low temperatures? Apparently not, as the following experiments show. Two sets of extracts were prepared, one from the eyes of shrimps darkened on a white background by warm water (37° C.) and the other from the eyes of shrimps darkened on a white background by cold water (15° C.), both groups being subjected to their respective temperatures for the same length of time, namely 45 minutes. Two sets of blinded shrimps were then selected, one set being injected with 0.1 cc. of one extract and the other set with the same amount of the other extract. These animals were then replaced in water at room temperature and the results noted. In both cases these darkened shrimps paled within the specified length of time, but with this difference,—the blue color previously described appeared in only one out of three of the shrimps injected with the extract prepared from the eyes of animals kept at low temperatures, while it appeared in all of the shrimps injected with the extract prepared from the eyes of animals kept at high temperatures. Neglecting for the present the significance of this variation, it is obvious that extreme temperatures in no way inhibit the manufacture or even the potency of the chromatophore-contracting hormone elaborated by the eye stalks.

This gives us a clue as to the manner in which heat and cold affect the chromatophores of crustaceans. Unfortunately, these experiments cannot give us a conclusive solution to this problem, though the data at hand strongly indicate a direct effect. Positive information is not to be derived from experiments on limbs or bits of integument isolated from the bodies of these shrimps, as the chromatophores of such excised pieces expand at once. Consequently subjecting such preparations to temperature variations accomplished no change in the distribution of their expanded chromatophore pigment. But since experiments on blinded and chloretonized shrimps give no evidence of any other type of response to temperature changes than those seen in normal light shrimps, and since neither heat nor cold affect the secretion of the chromatophore-contracting substances elaborated in the eye stalks, it seems reasonable to assume that the responses of the chromatophore pigment of crustaceans to high and low temperatures are direct.

A word or two in regard to the blue color and its relation to temperature changes. Keeble and Gamble (1903) state that the blue color observed in nocturnal *Hippolyte* disappears completely at 60° C., while, as shown in an earlier paper (Gamble and Keeble, 1900), this color is maintained at 8° C. under conditions that in other prawns kept at a somewhat higher temperature (15° C.) produce its loss. This latter observation is in accord with the experiments of Doflein (1910) on the occurrence of a blue color in *Leander* when the animals were kept for an extended period in darkness and cold. But aside from this, it is perhaps worthy of note that, as already mentioned, blinded animals injected with the extracts prepared from the eyes of shrimps subjected to cold showed only in one third of the cases a visible blue color, whereas blinded shrimps injected with an extract from the eyes of animals kept at high temperatures never failed to become pronouncedly blue. Furthermore, throughout the course of these experiments the blue color was repeatedly observed in connection with shrimps subjected to high temperatures, while the records disclose only one instance where it was seen in connection with shrimps exposed to low temperatures; a case where an animal kept at 6° C. for about 30 minutes turned blue when returned to 28° C. Perhaps this indicates a relationship between changes in temperature and the appearance of the blue color worthy of further investigation.

A survey of the work of previous investigators dealing with the action of heat and cold upon the crustacean chromatophore reveals a wide divergence of opinion. As we have already seen, Gamble and

Keeble (1900) claimed that both high and low temperatures produce or at least maintain a retraction of the pigment, a statement with which Jourdain (1878) and Doflein (1910) are in agreement as far as low temperatures are concerned. Menke (1911), on the other hand, reports that in *Idotea* extreme high and low temperatures tend to produce an expansion of the chromatophore pigment, though moderately high temperatures (20°–25° C.) lead to a contraction. Megušar (1911), however, observed an expansion of the chromatophore pigment with heat and a contraction with cold, though this author apparently did not subject his animal to temperatures lower than 15° C. In the most recent communication Koller (1927) maintains that temperature changes have no effect whatever upon the distribution of the chromatophore pigment in *Crangon.*

The results of the present writer's investigations are more in accord with those of Menke than with those of other workers, since Menke also observed an expansion of the pigment at both ends of the effective temperature scale. *Macrobrachium acanthurus* is a semi-tropical form, habituated to water normally remaining at 25°–30° C. the year around. Therefore, the response to temperature changes of such forms might reasonably be expected to vary somewhat from those seen by Menke in *Idotea,* a form adapted to life in cooler waters. Consequently we need not be greatly concerned when *Idotea* responds to temperatures of 20°–25° C. and Cuban shrimps do not. For the latter such temperatures are obviously not warm. The important feature is that for both types an expansion of the chromatophores is produced on exposure to temperatures either extremely high or extremely low.

Among the lizards and amphibians high temperatures as a rule produce a contraction of the chromatophores and low temperatures an expansion. Among the vertebrates in general, variations from this scheme are found in certain amphibians whose chromatophores are apparently insensitive to heat and among the fishes, where innervated melanophores react to warmth by expansion and to cold by contraction. The denervated melanophores of fishes respond, however, to temperature changes as do the chromatophores of lizards and amphibians. Since in these last two groups such reactions are presumably direct, and since they are certainly direct in denervated fish melanophores, it is permissible to say that among the vertebrates the independent response of the chromatophore to heat is a contraction and to cold an expansion. In the crustacean chromatophore where there is a high probability that reactions to temperature variations are direct, though this is admittedly not certain, an expansion of the chromatophore pigment is produced both by heat and cold. On the basis of our present knowledge then there seems to be little resemblance between the pigmentary reactions to heat and cold in the vertebrates and the crustaceans.

Among the vertebrates, especially in the lacertilians, the ability of the pigment cells to respond to temperature changes is sometimes given a thermo-regulatory significance. But the crustacean chromatophore can certainly serve no such purpose, especially as the chromatic responses of this group are controlled by factors other than heat and cold. It is inconceivable, for instance, that the form used in these experiments would ever encounter in its usual environment temperatures high enough or low enough to bring about changes in the distribution of its chromatophore pigment other than the distribution determined by background or light intensity.

SUMMARY

1. Expansion of the chromatophores of *Macrobrachium acanthurus*, a Cuban shrimp, follows immersion of these animals in fresh water at any temperature between 6° and 15° C. or between 35° and 40° C. This reaction occurs regardless of the background upon which the shrimp is placed. Between 15° C. and 35° C. the chromatophores of this shrimp expand when the animal is placed upon a black background and contract when the animal is placed upon a white background.

2. In blinded and chloretonized shrimps, the chromatophores are expanded and this expansion is in no way altered by changes in background or temperature.

3. Neither high nor low temperatures have any effect upon the potency or manufacture of the chromatophore-contracting substance elaborated by the eye stalks.

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UNUSUAL TYPES OF NEPHRIDIA IN NEMERTEANS

WESLEY R. COE

Osborn Zoölogical Laboratory, Yale University

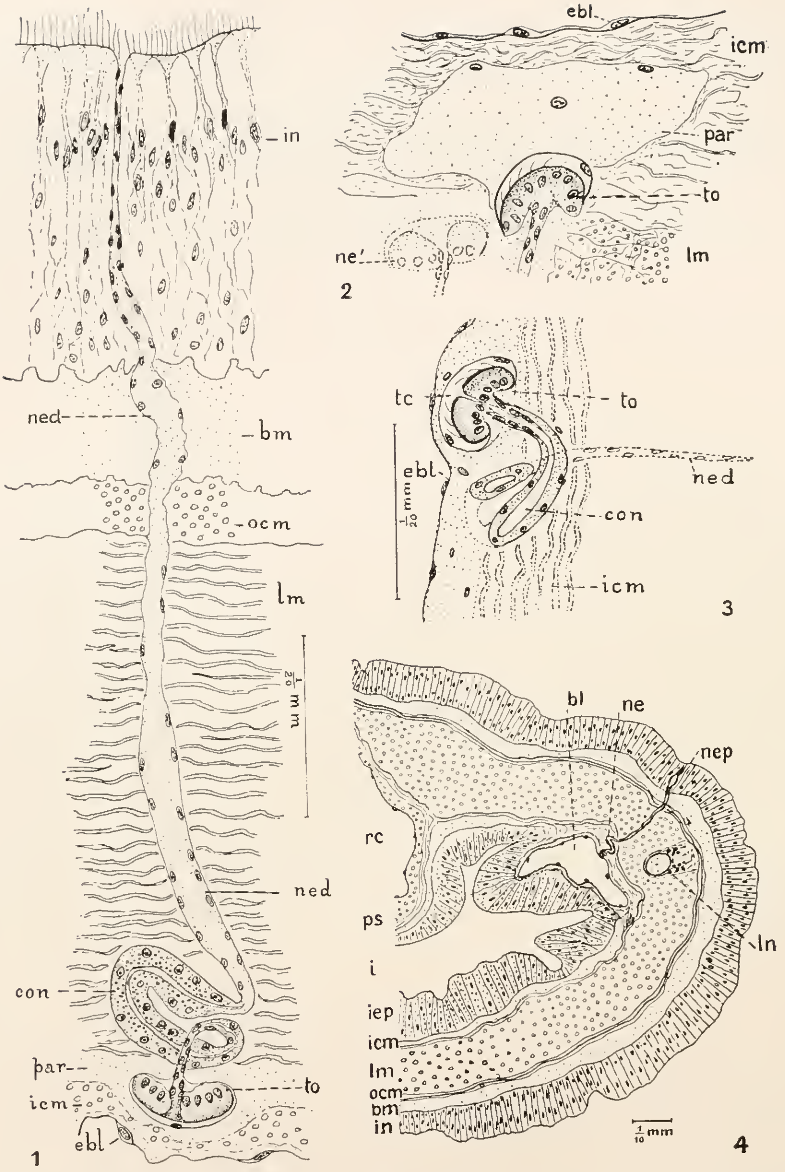
The earlier investigators on the morphology of the nemerteans failed to find in any of the species of the family Cephalotrichidæ the pair of longitudinal nephridial ducts which are so conspicuous in most nemerteans, and some of them came to the erroneous conclusion that in this family the nephridia are absent. Wijnhoff (1910) corrected this error, proving that a well-developed excretory system is actually present in the females of several species, but of a different nature than had been found up to that time in any nemertean. Instead of having all the terminal organs connected with a single longitudinal canal of comparatively large size, each end bulb has its own efferent duct leading to the exterior of the body.

Wijnhoff was unable to determine the exact configuration of the organs or the details of their histological structure, although she describes and figures the terminal organs in their relation with the lateral blood vessels and shows the groups of granular cells adjacent to the end bulbs.

Metanephridia in Cephalothrix Major

On the coast of California occurs a species of *Cephalothrix* (*C. major* Coe), in which the worms reach a size many times larger than those of other known species of the genus and in which the histological structure of the extremely complex excretory organs is clearly shown.

In this species, as in those studied by Wijnhoff, there is a series of isolated nephridia in close contact with the lateral blood lacunæ on each side of the body. Each nephridium consists of a multinucleate terminal organ, or end bulb, with slender flagella on its free border, and with a narrow canal leading to an enlarged glandular and convoluted tubule and thence by an efferent duct to a minute pore on the dorsolateral aspect of the body (Figs. 1, 4, 5, 8, 9).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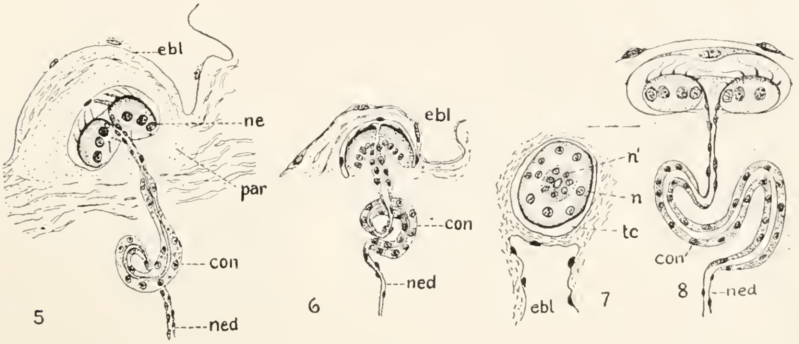
Figs. 1–4. Metanephridia of *C. major.* Fig. 1. Entire nephridium with widely opened efferent duct. Fig. 2. Terminal organ (nephrostome) associated with a large area of gelatinous parenchyma. Fig. 3. Terminal organ close beneath epithelial lining of blood lacuna. Fig. 4. Diagram of portion of body near anterior end of intestinal region, showing position of nephridium (*ne*) and efferent duct; *bl,* blood lacuna; *bm,* basement membrane of body wall; *con,* convoluted tubule; *ebl,* epithelium of blood lacuna; *icm, lm, ocm,* inner circular, longitudinal and outer circular musculatures; *iep,* intestinal epithelium; *in,* integument; *ln,* lateral nerve; *ned,* efferent duct; *nep,* nephridiopore; *par,* parenchyma; *ps,* proboscis sheath; *rc,* rhynchocoel; *tc,* terminal chamber; *to,* terminal organ.

The number of such independent nephridia is very large, more than 300 being found on each side of the body in an adult worm measuring a meter or more in length. All of them are found in the anterior half of the body. The most anterior ones border the blood lacunæ anterior to the mouth, the others being situated beside the lateral lacunæ in the region of the foregut and extending posteriorly beyond the anterior limits of the gonads. Although the nephridia are not paired on the two sides of the body, there is more or less regularity in their arrangement. Anteriorly they are more widely spaced than somewhat farther back, and they are most closely placed and of maximum size in the region where the foregut opens into the intestine, that is, in the region somewhat posterior to the most anterior gonads. More posteriorly they are not only farther apart, but are appreciably smaller and with fewer nuclei.

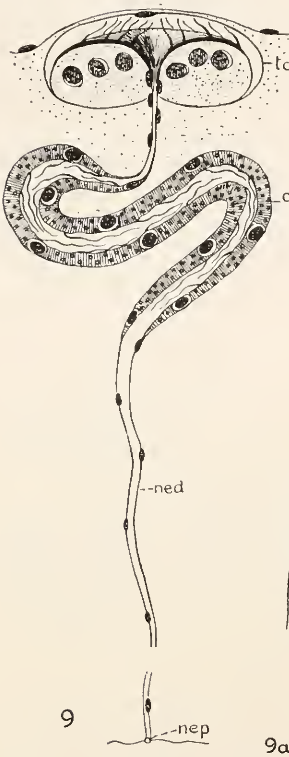


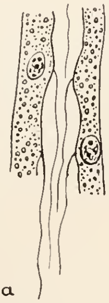
FIGS. 5–8. *C. major.* FIG. 5. Nephrostome (*ne*) imbedded in bulbous mass of gelatinous parenchyma (*par*). FIG. 6. Nephrostome close beneath epithelium of blood lacuna (*ebl*). FIG. 7. Transverse section of nephrostome, showing outer circle of nuclei (*n*) belonging to the flagella-bearing cells and the inner circle of smaller nuclei (*n'*) lining the end canal. FIG. 8. Diagram of nephridium in longitudinal section.

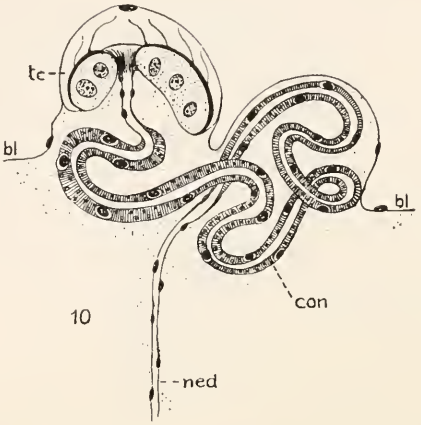
The actual distance between adjacent nephridia is commonly from 0.1 mm. to 0.2 mm. in the mounted sections, although some are separated by only 0.05 mm. or twice the diameter of the terminal bulb.

All are placed in a very similar situation with regard to the blood lacunæ and the nerve cords, always lying near the lumen of the blood space in the angle adjacent and somewhat dorsal to the nerve cord (Fig. 4). In many cases the terminal organ is situated on a low papilla, formed of the endothelium of the blood lacuna and its underlying basement membrane. This papilla projects somewhat into the lumen of the blood space, so that the greater part of the surface of the nephridium comes in close proximity to the blood (Figs. 5, 6).

Each nephridium consists of three principal parts, (*a*) the terminal bulb, (*b*) the convoluted tubule, and (*c*) the efferent duct (Figs. 1, 8, 17).







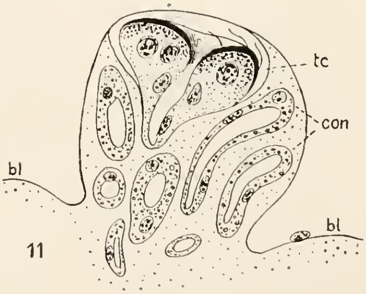


Fig. 9. Diagram of nephridium of *C. major*, showing slender flagella in lumen of convoluted tubule (*con*); 9*a*, small portion of convoluted tubule with flagella.

Fig. 10. Diagram of nephridium of *C. spiralis*, showing both nephrostome and convoluted tubule in bulbous projections on wall of blood lacuna (*bl*).

Fig. 11. *C. spiralis*. Section of nephrostome (*tc*) and loops of convoluted tubule (*con*) in single bulbous projection of wall of blood lacuna (*bl*).

(*a*) *The Terminal Bulb* (*Nephrostome*).—This lies in all cases in close proximity to one of the lateral blood lacunæ, which are usually much distended throughout the nephridial region (Fig. 4). Sometimes the bulb occupies a small papilla projecting somewhat into the lumen of the blood space and separated from the latter only by a thin covering of parenchyma and the endothelial lining of the blood vessel (Fig. 3). More often it lies deeper in the tissues and separated from the blood vessel and from the surrounding tissues by the mass of gelatinous parenchyma in which it is always imbedded (Fig. 2).

The terminal bulb is a mushroom-shaped structure with an extremely fine tubular stalk and with the free convex surface projecting into a hemispherical chamber (Fig. 3). Occasionally it can be demonstrated beyond question that a number of delicate flagella project freely into the terminal chamber and that the lumen of the latter is continuous with the slender tubule which pierces the stalk of the mushroom-shaped end bulb (Figs. 5, 8). The flagella are obviously projections from the free surface of the end bulb, and their vibration in life doubtless serves to draw into the tubule the fluid which collects in the vacuole.

The wall of the chamber consists of a very delicate membrane with one or two oval nuclei on its inner surface (Fig. 5). Two types of cells are found in the terminal organ, (*a*) those which compose the mushroom-shaped body and bear the flagella and (*b*) those belonging to the tubule of the stalk. In neither part are there distinct cell boundaries, but in the former the nuclei are much larger than in the latter (Figs. 5, 6). The cytoplasm on the hemispherical free surface of the terminal organ is dense and firm, forming a suitable support for the flagella. In the deeper part of this cytoplasm upwards of 20 oval nuclei are imbedded, six to eight of these being seen in a single longitudinal section (Figs. 5, 6, 8). In a cross section, however, the entire number may be shown (Fig. 7).

The canal in the tubular stalk is very slender and only in exceptional cases is the lumen demonstrable, due to the state of contraction at the moment of preservation. The nuclei of this canal are often only half the diameter of those of the terminal organ, or even less (Figs. 7, 8). Their number seldom exceeds a dozen. The size of the terminal bulb varies considerably as may be seen from figures 5–8, which are all drawn to the same scale, the transverse diameter being usually from 0.024 to 0.027 mm., although the smallest are only 0.018 mm. across and the largest as much as 0.03 mm.

(*b*) *The Convoluted Tubule.*—The slender canal in the stalk passes through the parenchyma surrounding the terminal bulb and then enlarges suddenly into a coiled tubule of much greater diameter and often with a conspicuous lumen (Figs. 1, 3, 9). This part of the nephridium is imbedded in a restricted mass of parenchyma more or less continuous with that surrounding the terminal bulb and extending into the inner portion of the longitudinal muscular layer. The configuration of the convolution is quite variable, as a comparison of the various figures will show. Sometimes there is but a single loop, but usually the tubule twists spirally in an irregular manner, parts of it appearing in four or more serial sections. The cytoplasm is coarsely granular, with numerous inclusions, but the nuclei are not separated by distinct cell boundaries (Fig. 9). This part of the nephridium closely resembles in structure the main longitudinal canal in those forms having compound nephridia (protonephridia), and its coarsely granular and vacuolated cytoplasm indicates that it has an important excretory function.

Long slender cilia project from the inner walls of the convoluted tubule; giving the appearance of fine threads lying lengthwise in the lumen and extending in the direction of the efferent duct (Figs. 9, 9*a*).

(*c*) *The Efferent Duct.*—The convoluted tubule leads directly into an extremely slender efferent duct which passes radially, that is, dorsally and laterally, in one of the connective tissue dissepiments separating the bundles of longitudinal muscles. It then pierces the outer circular musculature, the basement membrane and the integument, to open by a minute pore on the dorsolateral surface of the body (Figs. 1, 4, 9). The course of the duct may be so perfectly straight that nearly the entire length may be contained in one or two of the serial sections, but it is naturally seldom that the plane of the section coincides exactly with that of the duct.

The wall of the duct is extremely thin, but the cytoplasm bears numerous oval nuclei throughout its entire length. Even where the duct pierces the integument it has its independent nucleated lining (Fig. 1), as Wijnhoff (1910) has already demonstrated for other species.

Discussion

Excretory organs of this type have not been described for any of the other groups of Plathelminthes. In some of the Annelids, however, organs of somewhat similar structure are found, each with a ciliated funnel (nephrostome) opening into the body cavity and with a convoluted tubule, often of great complexity.

In the nephridium of *Cephalothrix* the mushroom-shaped end bulb is apparently homologous with the nephrostome of the annelid and may be so designated. The terminal chambers in *Cephalothrix* then represent minute cœlomic cavities, the fluid contents of which are in communication with the outside world through the nephridia, exactly as in annelids.

This type of excretory organ may be designated a metanephridium in order to distinguish it from the more usual type, protonephridium, found in nemerteans (Fig. 17, *B*), where each of the numerous end bulbs consists of a single flagellated cell imbedded in the body parenchyma and with its free border directed toward the efferent duct.

Physiology of the Metanephridium

The process of excretion by this type of nephridium is presumably accomplished by the withdrawal of waste-containing fluids from the surrounding gelatinous parenchyma, and thus indirectly from the nearby blood, by means of the ciliary action of the nephrostome. These fluids then pass to the convoluted tubule, the cells of which are specialized for the excretion of additional waste materials or for the absorption of any contained nutrients, or both; after which the remaining fluid is forced through the efferent duct to be discharged through the nephridiopore. The movement of the fluid in the convoluted tubule is doubtless facilitated by the slender flagella with which it is provided. The numerous granules and minute vacuoles in the cytoplasm of this part of the nephridium are indicative of its excretory function, as Strunk (1930) has recently demonstrated experimentally for Annelids.

Excretory Systems in Cephalothrix Spiralis

In another species of the genus, *C. spiralis* Coe (formerly considered specifically identical with *C. linearis* Oersted of Europe) of the New England coast, the excretory system of the female is likewise of the metanephridial type. In the two sexually mature males of this species which were available for study, however, no metanephridia were found, the excretory system consisting of a pair of clusters of protonephridia situated on the median walls of the cephalic blood lacunæ (Figs. 13, 15). The meaning of this apparent sexual dimorphism is by no means clear and will require further investigation on immature forms of both sexes. It may be remembered in this connection, however, that a somewhat similar condition prevails for the reproductive organs of some of the bathypelagic nemerteans, the males of which have only a few pairs of spermaries (and these are situated in the head), while the females are provided with numerous ovaries on each side of the body in the intestinal region (Coe, 1920). It will be recalled also that in the Annelids and other groups of invertebrates the larval excretory system is frequently of the protonephridial type, and is later replaced by the metanephridia. It seems possible that the sexual dimorphism in *Cephalothrix* may be similarly accounted for, assuming that the males have retained the primitive protonephridia, and that these are replaced in the females by the more complicated, and presumably more efficient metanephridia. Studies on immature individuals of both sexes will be made in the near future.

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METANEPHRIDIUM

The metanephridium of the female *C. spiralis* is similar to that of *C. major,* but is considerably larger in proportion to the size of the body and is more intimately associated with the lumen of the blood lacuna (Figs. 10, 11, 12, *D, E, F*). The average diameter of the nephrostome in this smaller species is about 0.023 mm., with some as small as 0.012 mm., as compared with 0.018 to 0.03 mm. in *C. major.*

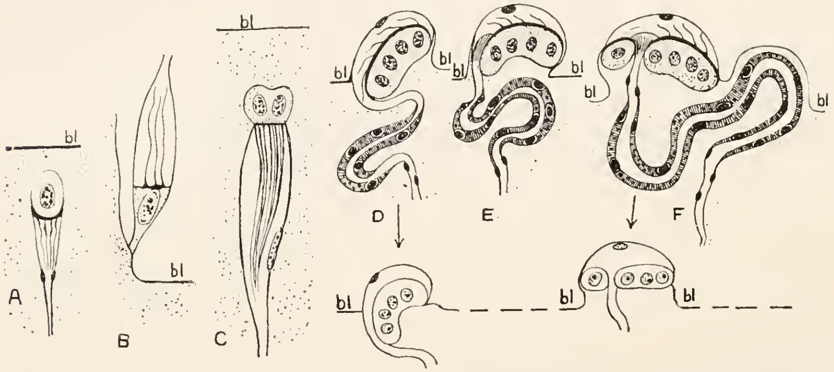


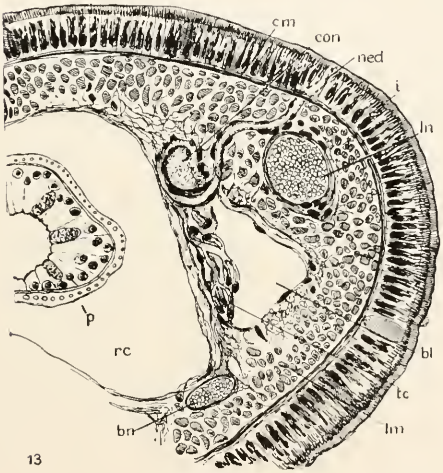
FIG. 12. Diagrams of the various types of nephridia found in nemerteans, showing the relation of each to the blood lacuna; *A,* protonephridium, characteristic of most nemerteans, imbedded in parenchyma close beneath blood lacuna; *B,* protonephridium of *C. spiralis,* male, hanging free in blood lacuna; *C,* protonephridium of *Geonemertes,* imbedded in parenchyma; *D, E, F,* metanephridia of *C. spiralis,* female, in successive stages of differentiation.

The shape as well as the position of the nephrostome varies considerably in the same individual. Only occasionally is the organ circular in surface view, with the opening of the end canal in the center. More often the opening is considerably eccentric, showing more nuclei on one side than on the other in vertical section (Figs. 10, 11, 12, *F*). In some cases the organ is heart-shaped or distinctly bilobed, with the opening in the indentation (Fig. 12, *D, E*).

In regard to their position relative to the blood lacunæ, both the nephrostome and the entire convoluted tubule may lie in the parenchyma beneath the epithelium and make no encroachment whatever on the lumen of the blood space or both may form bulbous projections into the lumen (Figs. 10, 11, 12, *D, E, F*). As a general rule, however, the terminal chamber projects freely into the blood space, while considerable gelatinous parenchyma lies between the convoluted tubule and the epithelial lining of the lacuna.

In the females a single pair of metanephridia is situated on the dorsolateral borders of the cephalic lacunæ not far anterior to the mouth. The convoluted tubule of this nephridium is greatly elongated anteroposteriorly, with the slender efferent duct at its posterior end. Anterior to the midgut the nephridia are widely scattered, increasing in abundance in the anterior portion of the gonad region and becoming less numerous beyond the end of the proboscis sheath. At least a hundred pairs are found in an individual of moderate size. In the mounted sections the distance between adjacent nephridia is usually 0.1 to 0.2 mm. in the anterior midgut region.

The nephrostome is frequently situated on a horizontal level with the lateral nerve cord, with the convoluted tubule either anterior or posterior and slightly dorsal thereto, but sometimes the nephrostome is found much nearer the ventral side of the body. In the latter case the efferent duct passes dorsally above the level of the nerve cord before leading radially to the nephridiopore on the dorsolateral surface of the body (Fig. 14).



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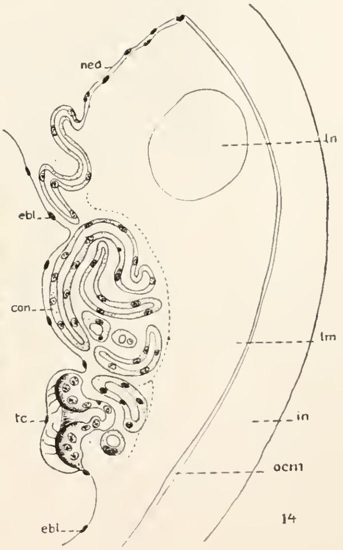

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FIG. 13. *C. spiralis.* Portion of transverse section through head of male, showing terminal chambers (*tc*) of nephridium on median wall of blood lacuna (*bl*); *con*, convoluted tubule opening to surface through efferent duct (*ned*): *bn*, buccal nerve; *cm* and *lm*, circular and longitudinal musculatures; *ln*, lateral nerve cord; *p*, proboscis.

FIG. 14. *C. spiralis.* An unusually large nephridium from the intestinal region posterior to the end of the proboscis sheath, showing the terminal chamber (*tc*) adjacent to the blood lacuna (*ebl*) and the voluminous convoluted tubule (*con*) leading dorsally to join the slender efferent duct (*ned*); *in*, integument; *ln*, lateral nerve; *lm*, *ocm*, longitudinal and outer circular musculatures.

The nephrostome is evidently capable of considerable change of shape by contraction and extension, for the mouth of the end canal joining the terminal chamber may be widely opened (Figs. 10, 11, 14) or it may be almost completely closed. The terminal chamber also may be distended with fluid and thus widely separated from the ciliated surface of the nephrostome (Fig. 10) or the fluid may be withdrawn, allowing the thin wall of the chamber to lie close upon the nephrostome.

A few double nephridia were found, and Wijnhoff (1910) observed the same condition in one of the species which she studied. The twinning may involve only the terminal organ and its accompanying end canal or it may include also the entire convoluted tubule. In the latter case two complete nephridia join a single efferent duct.

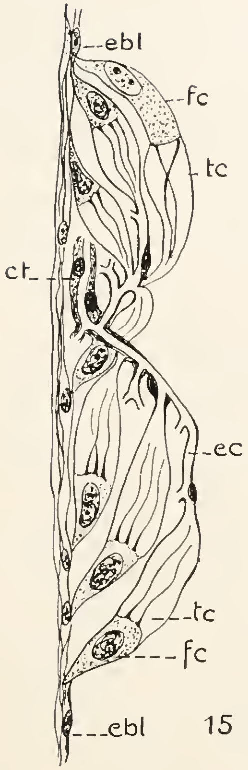
PROTONEPHRIDIUM

Mention has been made of the fact that metanephridia have been found thus far only in the females of the several species studied. Only two sexually mature males of *C. spiralis* with suitable fixation have been available for study and both of these were provided with excretory organs of the protonephridial type (Figs. 13, 15).

Each of the two individuals had a single pair of these organs situated on the median borders of the cephalic blood lacunæ between the brain and the mouth. Each nephridium consists of a cluster of fifty or more end organs connected with a branched collecting tubule which leads dorsally along the median face of the lacuna (Figs. 13, 15). On the dorsomedian angle of the lacuna the collecting tubule opens into the convoluted tubule, from which the efferent duct leads to the nephridiopore on the dorsolateral border of the head (Fig. 13).

Each of the end organs consists of a single cylindrical or goblet-shaped cell (flame cell) attached to the wall of the lacuna and more or less completely surrounded by the blood. The cytoplasm of the cell and the cell membrane are extended to form a central oval cavity in which the slender flagella may swing freely (Figs 12, *B*, 15). The proximal end of the flame cell is narrowed to a slender canal (end canal) which joins with others to form the collecting tubule.

Such an intimate association of flame cells with the blood is known for other species of nemerteans, but in no case is there any direct communication between the blood and nephridial systems. In order for fluid to pass from the blood to the excretory canal it must be filtered through the osmotic membranes and cytoplasmic extensions of the excretory cells.



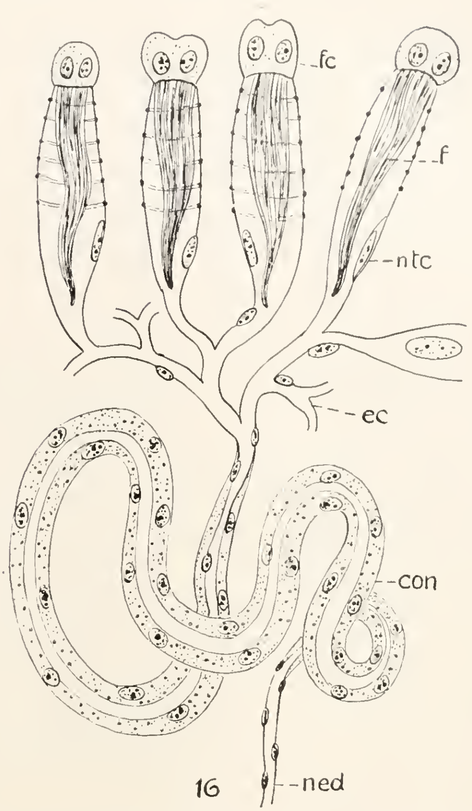


Fig. 15. *C. spiralis.* Diagram of cephalic protonephridium of male, showing the isolated flame cells (*fc*), with the terminal chambers (*tc*) leading to the slender end canals (*ec*) and thence to the collecting tubule (*ct*), convoluted tubule and efferent duct; *ebl,* epithelial lining of cephalic blood lacuna.

Fig. 16. *Geonemertes agricola.* Diagram of single nephridium with a cluster of slender terminal chambers (*tc*) and binucleate flame cells (*fc*) leading by the narrow end canals (*ec*) to a thick-walled convoluted tubule (*con*) and thence to the efferent duct (*ned*); *f,* tuft of long cilia; *ntc,* nucleus of terminal chamber; circular bars on wall of terminal chamber.

Comparison with Other Forms

With the exception of the metanephridia of the females of species belonging to the family Cephalotrichidæ, the excretory systems of all nemerteans in which such organs have been discovered are of the protonephridial type. In the numerous species of bathypelagic nemerteans, as well as in the littoral *Prosadenoporus,* no trace of an excretory system has yet been found.

Characteristic of the vast majority of species is a system of simple flame cells (Fig. 12, *A*; Fig. 17, *B*) imbedded in gelatinous parenchyma in close proximity to a blood space. Slender end canals from the flame cells lead to profusely branched collecting tubules and thence to a single thick-walled longitudinal canal on each side of the body. One or more slender efferent ducts lead from the longitudinal canal to the exterior of the body (Fig. 17, *B*). Occasionally, also, some of the efferent ducts open into the esophagus (Coe, 1906).

In such a system the ciliary action of the flame cells may withdraw fluids from the surrounding parenchyma and thence from the contiguous

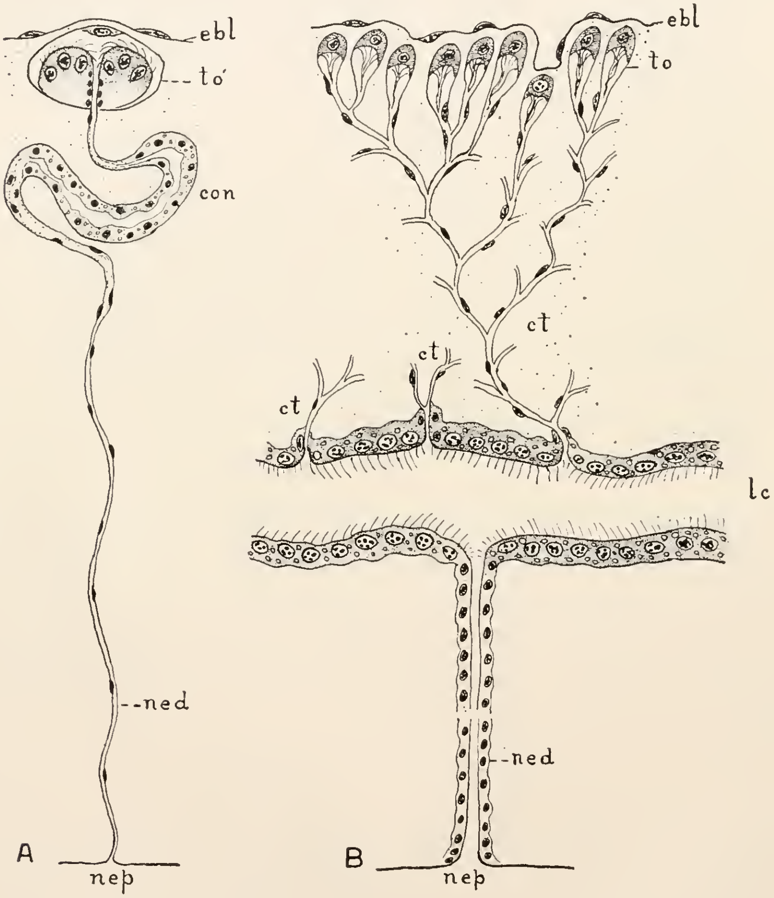


Fig. 17. Diagrams showing comparison between a simple metanephridium (*A*) of *Cephalothrix* and the multiple protonephridium (*B*) more typical for the nemerteans; *con*, convoluted tubule; *ct*, collecting tubule; *ebl*, epithelial lining of blood lacuna; *lc*, main longitudinal canal; *ned*, efferent duct; *nep*, nephridiopore, *to*, terminal organ.

blood space. After passing through the collecting tubules the fluid enters the longitudinal canal with its thick walls of granular and vacuolated cytoplasm, indicative of the secretory or excretory function of this part of the system. After receiving the contributions from the cells of the longitudinal canal, and possibly also returning to those cells any nutrient materials that it may contain, the fluid is discharged through the efferent ducts. The movement of fluids through the system is facilitated by the delicate cilia with which the longitudinal canal is provided (Fig. 17, *B*).

This system is commonly limited to the region of the body lying between the mouth and the midgut, where the blood spaces are voluminous and thin-walled, but in some cases it extends through other regions of the body. In the fresh-water *Prostoma,* for example, nephridia extend the entire length of the body, being separated into several independent groups in the adult, but connected together in early life.

In the terrestrial nemerteans, *Geonemertes,* there are many isolated groups of flame cells, each group with a convoluted tubule similar to the longitudinal canal in nature, and with its own efferent duct (Fig. 16). The number of such isolated nephridia may be very great and their extent may cover the greater part of the body. They are found not only in the vicinity of the lateral blood vessels but also in the parenchyma beneath the intestine and beside the proboscis sheath (Coe, 1929). As many as 35,000 are estimated to be present in one of the terrestrial forms which has a body length of only 35 mm. (Schröder, 1918). The terminal chamber in these forms is relatively large and its wall of much complexity (Figs. 12, *C*; 16).

Although the terminal organ of *Geonemertes* is composed of a binucleate flame cell and a cylindrical collar cell, we know of no transition stage between this protonephridium and the multinucleate metanephridium of the female *Cephalothrix.* And although the convoluted tubule of the latter is apparently homologous with the longitudinal canal of the protonephridium, the terminal organs of the two types seem to have originated independently, somewhat as have the larval protonephridia and the adult metanephridia of the Annelids.

But the question as to whether the metanephridium of *Cephalothrix* is preceded in the life history by an earlier excretory system of the protonephridial type remains at present unanswered.

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BLOOD SUGAR AND ACTIVITY IN FISHES WITH NOTES ON THE ACTION OF INSULIN

I. E. GRAY AND F. G. HALL

(From the Zoölogical Laboratory, Duke University)

The blood sugar of fishes has been studied by numerous investigators and great variations in amount have been reported for different species. In most cases a given observer has worked on one or a very few species, and correlations between the amount of sugar and the habits of the fishes have not been attempted. Furthermore, it is difficult to compare the results of different authors, since so many methods of determining blood sugar have been employed. Macleod (1926), has suggested that the more active fishes have higher blood sugar than do the more sluggish forms. One of us (Gray, 1929), has also pointed out a correlation between activity and blood sugar, but detailed data were not given. Among the mammals, Shirley (1928) hints at a tendency for low blood sugar to accompany high activity. She, however, did not make a comparative study, but limited her observations to a single species.

In a previous paper (Hall and Gray, 1929), a correlation was pointed out between the habits of marine fishes and their hemoglobin concentration. It was shown that among fifteen species of marine teleosts, in general, the most active had the highest iron values, while the blood of sluggish fishes had low iron content. The fishes with the highest iron content were surface feeding forms with similar habits, and fishermen consider them among the fastest swimmers. The highest hemoglobin was noted among members of the families Scombridæ and Clupeidæ, examples of which are, respectively, the mackerels and menhaden. These fishes feed largely on plankton and small fishes, which they can only obtain by constantly keeping in motion. At the other extreme are the bottom feeders, such as the goosefish, toadfish, and sand dab, which are very sluggish and have extremely low hemoglobin. These forms remain quiescent on the bottom for long periods of time. Between the two extremes are found the majority of fishes.

In the present paper it is shown that correlations similar to those between hemoglobin and activity exist also between blood sugar and activity.

This work was carried on at the United States Fisheries Station at Woods Hole, Massachusetts.

Materials and Methods

Blood sugar determinations were made on fifteen different species of teleosts, representing thirteen families, as shown in Table I. The fishes were obtained from commercial fish traps and were carefully placed in large floating "live cages," where they were kept free from asphyxial conditions for at least twenty-four hours before use. The importance of keeping the fishes free from asphyxial conditions cannot be overemphasized. In a previous paper (Hall, Gray, and Lepkovsky, 1926), the changes that take place in the concentration of the blood constituents of fishes under asphyxia were pointed out. Other workers (McCormick and Macleod, 1925; Simpson, 1926; and Menten, 1927) have noted that asphyxia tends to raise the blood sugar. The time required for fishes brought to the laboratory to recover from the partial asphyxia to which they have been subjected incidental to capture and transportation varies, of course, with the different species and with the methods of handling, both before and after they are placed in the "live cage." McCormick and MacLeod found that it required from two to four days for asphyxial hyperglycemia of the sculpin to subside. With our methods and facilities it was found that one full day was generally enough time to allow for recovery from any asphyxia to which the fishes might have been subjected. Menton (1927), concludes that the variation in sugar content of a species is governed largely by the amount of food ingested. In our experiments the food factor was reduced to a minimum by not using the fishes for a day or more after placing them in the "live cage."

The methods of procedure were similar to those employed in previous studies. The puffers, toadfish, and goosefish were bled from the heart with a hypodermic needle. The other fishes were bled by severing the tail and collecting the blood from the caudal vessels in a small Erlenmeyer flask. Lithium oxalate was used as an anti-coagulant. The blood sugar was determined by Folin's modification of the Folin-Wu method (Folin, 1926; Folin and Svedberg, 1926). A large percentage of the determinations was made on the same sample of blood used for the iron determinations (Hall and Gray, 1929), to which reference has previously been made. One fish was used for each determination.

During the study of the action of insulin the fishes were kept in hatchery boxes, one fish to each box. Insulin from Eli Lilly and Company was used throughout. The insulin was administered by intraperitoneal injections, in doses of five to fifteen units, depending on the size and species of fish. If the action of insulin in fishes is similar to its action in mammals, overdoses were given in each case.

TABLE I

The Blood Sugar of Marine Fishes

Species	Family	No. of Determinations	Sugar per 100 cc. of Blood		
			Low	High	Average
			Mg.	*Mg.*	*Mg.*
Group I					
Bull's eye mackerel (*Pneumatophorus colias*)	Scombridae	10	69.2	160.0	90.7
Butterfish (*Poronotus triacanthus*)	Stromateidae	8	57.5	113.6	79.4
Menhaden (*Brevoortia tyrannus*)	Clupeidae	30	52.9	151.5	75.2
Rudderfish (*Palinurichthys perciformis*)	Centrolophidae	7	54.9	83.3	67.7
Common mackerel (*Scomber scombrus*)	Scombridae	9	48.5	76.6	63.5
Eel (*Anguilla rostrata*)	Anguillidae	4	40.6	67.6	59.0
Bonito (*Sarda sarda*)	Scombridae	3	48.5	62.7	55.1
Scup (*Stenotomus chrysops*)	Sparidae	46	35.3	81.4	52.6
Silver hake (*Merluccius bilinearis*)	Merlucciidae	9	25.3	85.4	48.2
Group II					
Sea robin (*Prionotus carolinus*)	Triglidae	9	20.8	60.9	37.4
Sand dab (*Lophopsetta maculata*)	Pleuronectidae	4	24.6	42.5	31.0
Cunner (*Tautogolabrus adspersus*)	Labridae	4	13.4	35.1	25.2
Puffer (*Spheroides maculatus*)	Tetraodontidae	15	4.5	41.3	23.1
Toadfish (*Opsanus tau*)	Batrachoididae	6	10.2	22.3	15.4
Goosefish (*Lophius piscatorius*)	Lophiidae	11	0.0	10.3	5.6

RESULTS AND DISCUSSION

The results of the blood sugar determinations of the fifteen species of marine teleosts are given in Table I. The fishes were kept under conditions approximating the normal as nearly as possible. The high and the low blood sugar values are given together with the average to show the individual variation within the same species. The high and the low values may seem exceedingly far apart in a few cases, and without explanation may be misleading. The great majority of blood sugar determinations gave results near the average; it was only occasionally that a very high or exceedingly low value was obtained. There appears to be, however, a relatively greater individual variation among fishes of the same species kept under the same conditions, than among mammals.

Blood sugar, like hemoglobin, appears to be correlated in a general way with the habits and activity of the fishes. The bull's eye mackerel, butterfish, menhaden, rudderfish, common mackerel, eel, bonito, scup, and silver hake are not only more active fishes than the others given in the table, but also have higher blood sugar. For convenience of discussion the fishes are arbitrarily divided into groups I and II. There is no sharp dividing line, however, between the two groups.

Group I consists, for the most part, of aggressive fishes that depend on their own activities in obtaining food. They feed largely on plankton, small fishes, or other small animals that require the expenditure of considerable effort to obtain. Members of the Scombridæ and Clupeidæ are especially noted for their great activity. Individuals of these families are kept in captivity only with great difficulty even when placed in large " live cages " where they have plenty of room for their constant movements. It is doubtful if the bonito, mackerels, and menhaden ever cease their movements.

Some fishes of group I, for example, the scup and hake, might well be classed as intermediate in regard to their activity. They are not always in motion, nor do they pursue their food with the aggressiveness shown by the Scombridæ and Clupeidæ.

The fishes are arranged in the table, not in the order of their activity, but according to their blood sugar content. The correlation between activity and blood sugar is not absolute but occurs in most cases. If arranged according to relative activity the bonito would be at or near the top. The blood sugar of this species may not be strictly comparable with that of the other fishes since it was impossible to keep the bonito alive in captivity. Consequently the only data obtainable were determinations made on three small specimens, bled as soon as brought from the traps.

Some of group I are excellent migrating fishes that move rapidly through the water in large schools. Mostly they are adapted for fast movement by being " stream-lined " with body-form either fusiform or laterally compressed.

In group II are the relatively inactive and sluggish fishes. In contrast to the majority of group I, these fishes are the less aggressive bottom feeders that are adapted to life on the bottom by having the body-form angular or depressed. The cunner, although having a body-form resembling members of group I and being found in a variety of habitats, seems to prefer the rocky bottom and does not roam over wide areas in search of food.

It will be noted that the average blood sugar of the members of this group is considerably lower than that of group I. The goosefish and the toadfish are two of our most sluggish fishes and have very low blood sugar. Many determinations of the goosefish blood showed merely faint traces of sugar. The goosefish, although it feeds indiscriminately on other fishes, does not as a rule pursue its food. It is one of the anglers and attracts its prey by a lure on one of the dorsal fin-rays. All of the fishes of this group are known to remain quiet on the bottom for long periods of time, which habit is in sharp contrast to the activities of the mackerels and menhaden.

Table II

The Effect of Insulin on the Blood Sugar of Fishes

Species	No. of Determinations	Units of Insulin Given	Time for Shock to Appear	Sugar per 100 cc. of Blood	
				Normal	After Insulin
			Hours	*Mgs.*	*Mgs.*
Group I					
Menhaden	6	5	1½–3	75.2	8.6–20.2
Common mackerel	13	5	1–4	63.5	9.4–31.2
Bull's eye mackerel	5	5	3–6	90.7	9.4–11.1
Scup	20	5–10	10–23	52.6	0.0–15.3
Group II					
Sea robin	10	5–15	no shock	37.4	8.8–32.5
Puffer	9	5–15	no shock	23.1	0.0–13.5
Toadfish	15	5–15	no shock	15.4	1.5–22.9

We may say, then, that there appears to be a general correlation between the amount of sugar of the blood, the hemoglobin, the body-form, the activity, and the habits of marine fishes. Activity is expressed here qualitatively. There appears to be a dearth of quantitative determinations of metabolic activity in fishes. The oxygen consumption of the scup, puffer, and toadfish has been studied (Hall, 1929), and the results bear out our estimate of the activity of these fishes. Under the same conditions the oxygen consumption of the puffer was found to be intermediate between the relatively high consumption of the scup and the extremely low oxygen consumption of the toadfish. Because of their great activity a comparable basal oxygen consumption of the Scombridæ and Clupeidæ, fishes more active than the scup, could not be determined.

A further interesting relation to activity was noted through a comparative study of the action of insulin on fishes. At a temperature of 21° C. and under similar conditions, it was found that the very active fishes, menhaden, common mackerel, and bull's eye mackerel, showed insulin shock in a much shorter time than did the moderately active scup. This was perhaps to be expected. Huxley and Fulton (1924), and Olmsted (1924), have pointed out that the rate of action of insulin is dependent upon the metabolic rate of the animal itself. The more sluggish bottom feeders, sea robin, puffer, and toadfish, showed no external evidences of the effects of insulin. As has been previously noted, the normal blood sugar of these sluggish fishes is much lower than that of the more active ones. In some cases, such as the toadfish, the normal sugar concentration is not as high as the insulin-reduced sugar concentration of the more active fishes. A condensed summary of the action of insulin on fishes is given in Table II.

Insulin appears to reduce the blood sugar concentration of fishes in much the same manner as in mammals, except that a longer time is required for the action to take place. Although the number is limited, at least some of each species whose blood was analyzed showed reduced sugar concentration following insulin administration. The mackerels, menhaden, and scup, if bled during convulsions, showed reduced sugar content in each case. There is considerable individual variation in the time required for the sugar content to be reduced; and since the sluggish fishes showed no convulsions, it was difficult to estimate the length of time to allow for insulin action. Puffers, sea robins, and toadfish, bled at various intervals between twenty and forty hours after insulin injection, showed blood sugar values ranging from the normal to mere traces. Since some of each of these species showed reduction of sugar content, it is thought that in those cases where, after insulin administration, the blood sugar was within the normal range of variation, either enough time had not elapsed for the insulin to reduce the sugar concentration, or else too much time elapsed and the fishes regained the normal sugar content.

The time required for the blood sugar content to be reduced appeared to be considerably greater in these sluggish forms than in the more active fishes. It seems improbable that the failure to get insulin shock could be due to insufficient insulin. Toadfish were given repeated injections of from five to fifteen units of insulin over a period of several days with no visible signs of disturbed metabolism. With the mackerels, menhaden, or scup a single five unit injection usually resulted in death unless glucose was administered.

Insulin convulsions in fishes do not necessarily indicate that the blood sugar concentration is reduced to its lowest level. The rate of reduction of the blood sugar values following insulin injection has been worked out for the scup and will be published later. Here it is sufficient to say that the blood sugar content may be reduced in six to eight hours in this fish. In a few cases mere traces of sugar remained in the blood after eight hours and yet in no case were convulsions apparent sooner than ten hours. In other words, a few scup had lower blood sugar before reaching the convulsive stage than did other scup in the midst of convulsions. Furthermore, the fact that sluggish fishes, as the toadfish and puffer, have their blood sugar concentration reduced without showing any shock at all, indicates that insulin shock in fishes does not have as much significance as has been attributed to insulin convulsions in mammals.

Summary

1. Correlations between the blood sugar, hemoglobin, body-form, activity and habits of fifteen species of marine teleosts are pointed out.

2. The fishes that show the greatest activity, those that feed at the surface or are aggressively predaceous, have the highest blood sugar concentration. The sluggish bottom feeders have low sugar content in the blood.

3. Insulin shock may be easily produced in active species of fishes. In sluggish forms no external evidence of the action of insulin could be detected.

4. The blood sugar of fishes is reduced by the action of insulin. Less time is required for reduction of sugar content to take place in the active fishes than in the sluggish forms, due probably to differences in the metabolic rate of the different species. In the sluggish forms the sugar content may be reduced without convulsions or shock being apparent.

5. The normal sugar of some of the sluggish fishes is often lower than the insulin-reduced sugar of the more active fishes.

STUDIES OF PHOTODYNAMIC ACTION

I. Hemolysis by Previously Irradiated Fluorescein Dyes

HAROLD F. BLUM

Department of Physiology, Harvard Medical School[1]

The hemolysis of red blood cells by the combined action of light and certain photoactive substances was first described by Sacharoff and Sachs in 1905. Such hemolysis occurs in a very short time when red blood cells are exposed to sunlight in dilute concentrations of the photoactive substance. Sunlight alone does not produce hemolysis provided the ultra-violet spectrum is screened out by exposing the cells in glass, nor does the photoactive substance in equal concentration in the dark. This is only one of a wide range of similar phenomena brought about under similar conditions in other cells and tissues, which are generally described collectively under the term *photodynamic action* or *photodynamic sensitization.* The photoactive substances which bring these phenomena about include a large number of compounds, most of which are fluorescent dyes. It is generally assumed that such effects are not produced if the solution of the photodynamic substance is separately irradiated, the erythrocytes or other cells being added subsequently in the dark (see Clark 1922, p. 288). There are, however, a few recorded experiments which indicate that this is possible.

Ledoux-Lebard (1902) found that eosine which had been previously exposed to sunlight killed and cytolyzed paramecia; whereas non-irradiated eosine of the same concentration did not. He suggested, therefore, that photodynamic action is due to the formation of a toxic eosine compound by the action of sunlight. Jodlbauer and Tappeiner (1905) found that this did not occur if the eosine solution was neutralized after irradiation and before the addition of the paramecia. They claimed, therefore, that the Ledoux-Lebard effect was due to the formation of acid concomitant with the bleaching of the dye; the acid being the toxic agent. They did not consider this as a photodynamic effect. Sacharoff and Sachs (1905) described hemolysis by previously irradiated β-(o-nitrophenyl)—β-hydroxyethyl methyl ketone ["o-Nitrophenylmilchsäureketon"]. They were unable to produce hemolysis with previously irradiated eosine or erythrosine, however, and preferred to consider their one positive result as not belonging to the typical photodynamic phenomena. Fabre and Simonnet (1927) were able to produce hemolysis with lecithin which had been irradiated together with hematoporphyrin by light from a mercury vapour arc. Moore (1928) found that previously irradiated eosine killed the eggs of the sea urchin *Strongylocentrotus purpuratus* but did not cytolyze them; whereas when the eggs were irradiated together with the dye, they were completely cytolyzed. Moore hypothecates the formation of a toxic eosine compound which produces cytolysis upon further irradiation after it has entered the cell. On the other hand, Raab (1900) was unable to produce killing of paramecia by previously irradiated acridine solutions. Hausmann was unable to produce killing of paramecia or hemolysis with previously irradiated solutions of chlorophyll (1909) or hematoporphyrin (1910); although similar solutions produced these effects when irradiated together with the cells. Hasselbach (1909) could not produce hemolysis with previously irradiated solutions of several photodynamically active substances including eosine and erythrosine. Pereira (1925) found that *Arbacia* larvæ were not killed by previously irradiated eosine in sea water.

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[1] Preliminary experiments for these studies were carried out in the Department of Animal Biology, University of Oregon.

The writer has found that it is possible, under carefully controlled conditions, to bring about hemolysis with previously irradiated solutions of the three fluorescein dyes which he has investigated, fluorescein, eosine and erythrosine. This is of considerable interest because of its bearing on certain theories of photodynamic action which will be discussed later in this paper.

Experimental

Hemolysis by previously irradiated fluorescein, eosine, and erythrosine.—The writer's first attempts to produce hemolysis with previously irradiated eosine solutions met with apparent success in only a few instances. These were thought at first to be accidental, but with more careful control of conditions it was found possible to obtain consistently reproducible results. The successful technique required the selection of proper hydrogen ion concentration and dye concentration.

The hydrogen ion concentration must be carefully buffered, since unbuffered solutions tend to increase in acidity during irradiation. This increase in acidity may inhibit the production of hemolysis by bringing about fixation of the cells as will be pointed out in a later paper. To insure the maximum obtainable buffering capacity, it was found convenient to make up the dye in solutions of primary and secondary sodium phosphate mixtures. In order to insure a medium of proper osmotic pressure for the blood cells, the phosphate mixtures were calculated to have the same osmotic pressure as a 0.15 M sodium chloride solution. This was done by assuming that the primary phosphate dissociates into two ions, the secondary phosphate into three. The mol fractions of the two salts required for a given hydrogen ion concentration were estimated by the use of Cohn's data for potassium phosphates (see Clark, 1928, pp. 216–220).[2] The hydrogen ion concentrations of the solutions were checked by means of the hydrogen electrode. Such solutions proved rather unsatisfactory in the case of fluorescein, and a solution containing 10 per cent of the phosphate mixture and 90 per cent 0.15 M sodium chloride, was used instead in most experiments with this dye. The concentration of phosphate in this solution is still many times that of fluorescein in most of the dye concentrations which were used, and affords an adequate buffer.

The optimal concentration of the dye varies with a number of conditions; some of which, as for example the intensity of irradiation, it was impossible to control. It was found expedient, therefore, to use a series of dilutions of the dyes; usually consisting of ten dilutions from 1 per cent to 0.002 per cent.[3] These were exposed to the sunlight for a given period of time. Blood cells were then added to the irradiated solutions and also to a control consisting of a corresponding series of non-irradiated dye solutions. Both series were then placed in a dark room where the temperature was in the region of 20° C. Observation of the tubes for hemolysis was made at intervals after the addition of the cells. It was found that in most cases six hours sufficed for the hemolysis to reach a maximum. Since the temperature during irradiation could not be controlled, time was allowed, when necessary, for the irradiated tubes to come to the same temperature as the controls before adding the cells. The solutions were exposed in small test tubes (10 x 75 mm.), each containing 2 cc. of the solutions. The blood cells were added to each tube in the quantity of 0.02 cc. of a 50 per cent suspension in 0.15 M sodium chloride, by means of a blood pipette. This method avoids any appreciable dilution of the irradiated solution upon the addition of the cells. This precaution has not been observed by most of the investigators who have attempted to produce hemolysis with previously irradiated substances. Human blood cells were used in most of the experiments. They were washed by centrifuging three times from suspension in 0.15 M sodium chloride to free them from serum. It is advisable to have the cells as free from serum as possible, since serum is effective in preventing photodynamic hemolysis (Busck, 1906). The intensity of the radiation could not be accurately estimated, but it was found practicable to expose the solutions to bright midday sunlight for one to two hours. Too long continued exposure causes bleaching of the dye, resulting in a lowered concentration of the active dye.

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[2] Dr. G. Payling Wright and the writer have found that rabbit blood cells suspended in such solutions show a variation in volume of approximately twelve per cent over the range of hydrogen ion concentration between pH 7.7 and pH 6.0, and have approximately the same volume as cells in serum.

[3] The dyes used were Fluorescein, sodium salt (Uranine), from the National Aniline and Chemical Company, Erythrosine B (sodium salt of tetra-iodo-fluorescein) also from the National Aniline and Chemical Company, and Eosine Y (sodium salt of tetra-brom-fluorescein) from Coleman and Bell.

TABLE I

Hemolysis by Previously Irradiated Fluorescein

Solutions exposed to sunlight 90 minutes (2:00–3:30 P.M., September 29, 1929). All solutions contain 10 per cent of sodium phosphate buffer, pH 6.4, isosmotic with 0.15 M NaCl, plus 90 per cent of 0.15 M NaCl. Observations made after 16 hours in dark following addition of red blood cells. *H* = complete hemolysis, (*H*) = partial hemolysis, and the dash is used when there is no detectable hemolysis.

Concentration of fluorescein	Irradiated solution. Red blood cells added after		Non-irradiated solution (control)
per cent	45 minutes	4 hours	
1.0	—	—	—
0.5	—	—	—
0.25	—	—	—
0.125	(H)	—	—
0.062	(H)	(H)	—
0.031	H	(H)	—
0.015	H	H	—
0.007	(H)	(H)	—
0.004	(H)	(H)	—
0.002	—	—	—
0.00	—	—	—

Tables I, II, and III show the results of typical experiments with fluorescein, eosine and erythrosine respectively. In these tables, *H* represents complete hemolysis (*i.e.* hemochromolysis and stromatolysis) as well as can be judged by the naked eye, (*H*) represents partial hemolysis, and the dash no detectable hemolysis. These classifications are arbitrary, but since comparison can always be made with the control tubes, there can be no doubt of the general validity of the observations. An examination of Tables I, II, and III demonstrates quite clearly that previously irradiated solutions of these dyes bring about hemolysis in concentrations at which non-irradiated solutions do not. Some bleaching of the dye takes place upon irradiation and this raises the question whether the hemolysis may not be due to the products of this bleaching.

It has been found, however, that completely bleached solutions have no hemolytic action.

Non-irradiated eosine and erythrosine produce hemolysis in sufficiently high concentration, as is shown in Tables II and III. This was described by Sacharoff and Sachs (1905) and studied by Tappeiner (1908). It is apparently not due to irradiation during the preparation of the solutions; since in these experiments the results were the same when the solutions were carefully prepared in the dark room under red light, which is outside the absorption range of these dyes, as when prepared with ordinary precautions in the diffuse light of the laboratory. The effect of short exposure to diffuse light is thus within the accuracy of the observations described here. The absence of hemolysis in the higher concentrations of the irradiated dye in Table I is probably due to fixation of the cells. This phenomenon will be discussed in a later paper. The marked effect of hydrogen ion concentration upon the hemolytic activity of irradiated and non-irradiated dyes will also be discussed in that paper; the hydrogen ion concentrations for the experiments here described have been chosen as those at which the difference in hemolytic activity between previously irradiated and non-irradiated solutions could be most clearly demonstrated.

TABLE II

Hemolysis by Previously Irradiated Eosine

Solutions exposed to sunlight for 105 minutes (11:45A.M.–1:30 P.M., September 10, 1929). All solutions contain sodium phosphate buffer, pH 7.0, isosmotic with 0.15 M NaCl. Observations made 5 hours after addition of red blood cells. The symbols are the same as those in Table I.

Concentration of eosine	Irradiated solution. Red blood cells added after			Non-irradiated solution (control). Red blood cells added after	
per cent	45 minutes	2¾ hours	5 hours	45 minutes	5 hours
1.0	H	H	H	H	H
0.5	H	H	H	(H)	(H)
0.25	H	H	H	—	—
0.125	H	H	H	—	—
0.062	H	H	H	—	—
0.031	H	(H)	H	—	—
0.015	(H)	(H)	(H)	—	—
0.007	—	—	—	—	—
0.004	—	—	—	—	—
0.002	—	—	—	—	—
0.0	—	—	—	—	—

It will be noted in Tables I, II, and III that the results are changed very little when the exposed solutions are allowed to remain in the dark for as much as four or five hours after irradiation, before the addition of the cells. This shows very conclusively that the increased hemolytic activity of the irradiated solutions cannot be due to their having a greater temperature than the controls because of the absorption of heat during the period of exposure, since ample time is allowed for the two series of solutions to come to the same temperature. It also demonstrates that whatever change occurs in the course of irradiation is not rapidly reversible in the dark. Moore (1928) observed, similarly, that in the case of the killing of sea urchin's eggs by previously irradiated eosine, the solution retained its toxic properties after six hours in the dark.

Table III

Hemolysis by Previously Irradiated Erythrosine

Solutions exposed to sunlight for one hour (11:00 A.M.–12:00 M., September 28, 1929). All solutions contain sodium phosphate buffer, pH 6.5, isosmotic with 0.15 M NaCl. Observations made 6 hours after addition of red blood cells. The symbols are the same as those in Tables I and II.

Concentration of erythrosine	Irradiated solution. Red blood cells added after			Non-irradiated solution (control)
per cent	45 minutes	1¾ hours	5 hours	
1.0	H	H	H	H
0.5	H	H	H	H
0.25	H	H	H	H
0.125	H	H	H	H
0.062	H	H	H	(H)
0.031	H	H	H	(H)
0.015	(H)	(H)	(H)	—
0.007	—	—	—	—
0.004	—	—	—	—
0.002	—	—	—	—
0.00	—	—	—	—

Discussion

Numerous hypotheses have been developed to explain the mechanism of photodynamic action, most of which contain the assumption that the photodynamic substance and substrate (*e.g.* cells) must be irradiated together. This is true of the theory of Tappeiner (1909) which he outlines as follows: The presence of the photodynamic substance merely accelerates the action of visible light. The split products of this reaction are removed through oxidation by molecular oxygen. Ordinarily

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these products accumulate and inhibit the reaction, but the combined action of light and a photodynamic substance accelerates their removal and consequently the total reaction. Another conception, based on the fact that most of the photodynamic substances are fluorescent, is that the photodynamic effects are due to the action of fluoresced radiation upon the protoplasm. Since the fluoresced light is only a more or less polarized radiation from a particular region of the visible spectrum characteristic of the substance concerned (Pringshein 1928, p. 195), it can hardly be expected to have such destructive effects. Moreover, Raab (1900) showed that paramecia are not damaged when exposed to the fluoresced radiation from a solution of fluorescent substance with which they are not in contact; and likewise, Sacharoff and Sachs (1905) showed that red blood cells exposed under the same conditions are not hemolyzed. Nevertheless, this concept remains current to a certain extent. Schanz (1921) suggests from studies on the photoelectric effect in albumin, and albumin plus fluorescein dyes, that the changes brought about in the cell constituents are due to the absorption of electrons emitted by the dye during irradiation. Clark (1922, pp. 302–303) suggests that the photodynamic substance shifts the photoelectric threshold of the cell constituents from the ultra-violet into longer wave lengths. Metzner (1924) claims that the photodynamic effects are brought about by an action within the cell dependent upon the combination (adsorption) of the dye with the protoplasm. Jodlbauer (1926) assumes that the dye must be adsorbed by the cell, and that only those dyes are photodynamically active which retain their ability to be activated by light while in combination with the cell substance. Such theories demonstrate how firmly the idea is established that the photodynamic substance and substrate must be irradiated together. Obviously all such explanations of photodynamic action must be discarded or modified, in light of the fact that hemolysis may be brought about by previously irradiated photodynamic substances.

Experimental

Evidence that Oxidation Is a Factor in Photodynamic Hemolysis.— A theory of direct oxidation of cell constituents by the action of light and the photodynamic substance was put forward by Straub (1904*a*). His hypothesis was founded principally upon the analogy between the photodynamic action of eosine upon cells and its ability to oxidize iodide ion in the presence of light. He found (1904*b*) that, in proper concentration, eosine may oxidize many times its equivalency of iodide when the two substances are exposed to sunlight together in solution. He conceived that the eosine is changed to an eosine peroxide by the action of light; and that this peroxide brings about the oxidation of an equivalent amount of iodide ion, being returned in so doing to the original eosine form. The eosine may then proceed to the oxidation of another quantity of iodide, thus acting in a sense as a catalyst. He could not, however, demonstrate the existence of an intermediate peroxide, being unable to obtain conclusive evidence of the oxidation of iodide ion by the action of previously irradiated eosine (1904*a*).

The writer finds that previously irradiated eosine will oxidize iodide ion, as shown by a positive starch reaction after adding potassium iodide in the dark. The oxidation proceeds rather slowly immediately after the addition of the potassium iodide, which may account for Straub's failure to observe it in his experiments. Table IV presents some quantitative results obtained when (1) fluorescein dyes and potassium iodide were irradiated in solution together, and (2) when the iodide was added to the previously irradiated dyes. The determinations were made by titration of the free iodine formed due to the oxidation of iodide ion, with 0.001 N sodium thiosulfate against starch indicator. When potassium iodide is added to the previously irradiated dye and the mixture placed in the dark, the oxidation takes place quite slowly, reaching a maximum after about three hours. The titrations were, therefore, performed after the elapse of this time. The accuracy of determination of iodine in such small concentrations is, of course, subject to some error. In order to determine the magnitude of this error, solutions containing quantities of iodine of the same order as those represented in Table IV were titrated. The solutions were of the same volume, contained the same concentration of dye and of potassium iodide, and were buffered at the same hydrogen ion concentration as the experimental solutions. With concentrations of iodine corresponding to the lowest values in Table IV, the determinations were consistently 10 to 15 per cent lower than the theoretical. With quantities of iodine corresponding to the highest values the error was not greater than one per cent. The 0.001 N thiosulfate solution was always freshly prepared by dilution from a 0.1 N stock solution.

The experiments described in Table IV represent conditions in the region of the optimal for the reaction of the iodide with each dye. The extent of these reactions seems to be greatly affected by the hydrogen ion concentration, and by other factors, which will not be discussed here. Controls containing the same concentration of potassium iodide, but no dye, never showed more than a trace of free iodine when exposed to sunlight simultaneously with the potassium iodide-dye mixtures. Likewise, solutions of the dye containing potassium iodide showed no trace of free iodine after many hours in the dark.

TABLE IV

Oxidation of Potassium Iodide by Irradiated Fluorescein, Eosine, and Erythrosine

Fluorescein	KI	pH	Volume of Solution	Duration of Irradiation	KI Added after Irradiation	Volume of $Na_2S_2O_3$ 0.001N	Mols of Dye	Mols of Iodide Oxidized
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.0005M	1.0	6.0	6.0	8	0.0	18.6	3×10^{-6}	18.6×10^{-6}
0.0005M	1.0	6.0	6.0	8	0.0	17.1 *	3×10^{-6}	17.1×10^{-6}
0.0005M	1.0	6.0	6.0	0	0.0	0.0	3×10^{-6}	0.0
0.0005M	0.0	6.0	6.0	8	3.0	1.0 †	3×10^{-6}	1.0×10^{-6}
0.0005M	0.0	6.0	6.0	8	3.0	0.5 ‡	3×10^{-6}	0.5×10^{-6}
0.0	1.0	6.0	6.0	8	0.0	0.4	0.0	0.4×10^{-6}
Eosine								
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.001M	3.0	6.0	6.0	6	0.0	14.5	6×10^{-6}	14.5×10^{-6}
0.001M	3.0	6.0	6.0	6	0.0	14.5 *	6×10^{-6}	14.5×10^{-6}
0.001M	3.0	6.0	6.0	0	0.0	0.0	6×10^{-6}	0.0
0.001M	0.0	6.0	6.0	6	3.0	3.2 †	6×10^{-6}	3.2×10^{-6}
0.001M	0.0	6.0	6.0	6	3.0	3.0 ‡	6×10^{-6}	3.0×10^{-6}
0.001M	0.0	6.0	6.0	0	3.0	0.0	6×10^{-6}	0.0
Erythrosine								
	per cent		*Cc.*	Hours	*per cent*	*Cc.*		
0.001M	3.0	6.0	6.0	6	0.0	19.1	6×10^{-6}	19.1×10^{-6}
0.001M	3.0	6.0	6.0	6	0.0	19.5 *	6×10^{-6}	19.5×10^{-6}
0.001M	3.0	6.0	6.0	0	0.0	0.0	6×10^{-6}	0.0
0.001M	0.0	6.0	6.0	6	3.0	1.3 †	6×10^{-6}	1.3×10^{-6}
0.001M	0.0	6.0	6.0	6	3.0	1.0 ‡	6×10^{-6}	1.0×10^{-6}
0.001M	0.0	6.0	6.0	0	3.0	0.0	6×10^{-6}	0.0

* Titration after 3 hours in dark.
† KI added immediately after irradiation with titration after 3 hours in dark.
‡ KI added after 3 hours in dark following irradiation; titration 3 hours later.

Table IV shows that iodide ion equivalent to several times the quantity of dye present may be oxidized when exposed together with the dye (equivalency considered as one mol of iodide ion per mol of dye). Straub (1904*b*) was able, in fact, to oxidize a quantity of iodide ion sixty-five times as great as the quantity of dye present. On the other hand, when the dye alone is irradiated and the potassium iodide added subsequently in the dark, the quantity of iodide ion oxidized is always less than that equivalent to the dye present. In the latter case it was never found possible, in a considerable number of experiments under varying conditions, to oxidize more iodide than a quantity equivalent to the quantity of dye present. When irradiated in the absence of a readily oxidizable substance, such as iodide ion, a certain amount of the dye is oxidized, as is indicated by bleaching. Thus the transformation of all the dye to the active form cannot be expected, and we should expect that less iodide would be oxidized than a quantity equivalent to the quantity of the dye originally present. This appears to be the case. When the dye is exposed with a readily oxidizable substance, no bleaching occurs, indicating that this substance is oxidized instead of the dye. All these facts lend support to Straub's hypothesis. They demonstrate at least that a substance is formed upon irradiation of the dye solution which is capable of oxidizing substances which the non-irradiated dye cannot, and indicate that this is an intermediate substance in the oxidations brought about by the action of the dye and light.

The quantity of iodide ion oxidized is not greatly altered if the dye is allowed to remain in the dark for several hours after irradiation before potassium iodide is added. This shows that the change brought about by irradiation is not rapidly reversible in the dark. This is exactly parallel to the case of hemolysis where, as we have seen, hemolysis is brought about by previously irradiated dye solutions which have remained in the dark for several hours after irradiation before addition of the cells. Substances produced in the bleaching of the dye are not responsible for the oxidation of iodide ion, since completely bleached solutions do not bring about this oxidation. This is again parallel to the case of hemolysis, since as stated above, hemolysis is not produced by completely bleached dyes. These latter facts suggest very definitely that the substance in irradiated solutions of a fluorescein dye which brings about hemolysis is the same as that which brings about the oxidation of iodide ion; and that, therefore, the former process is probably dependent upon an oxidation.

If it is true that the hemolysis of blood cells by irradiated dyes involves the oxidation of cell constituents in a manner similar to the oxidation of iodide ion, we should expect, parallel to the above observations, more extensive oxidation and thus greater hemolysis when the dye is irradiated together with the cells than when previously irradiated. In the former case the dye may, presumably, act in a catalytic sense, thus oxidizing several times its molecular equivalency of cell constituents; whereas in the latter case the amount of oxidation is limited by the quantity of dye present. The data presented in Tables V, VI and VII, appears to confirm this prediction; the hemolytic action seems to be quantitatively much greater when the dye and cells are irradiated together than when the dye is irradiated alone and the cells added later in the dark. The statement that hemolysis is more readily produced

TABLE V

Comparison of Hemolytic Activity of Fluorescein Irradiated With and Without Blood Cells

Solutions exposed to sunlight for one hour and 30 minutes. All solutions contain sodium phosphate buffer, pH 6.5, isosmotic with 0.15 M NaCl. Observations made after 20 hours in dark following addition of blood cells. Symbols as in preceding tables. *P* = precipitate.

Concentration of Fluorescein	Fluorescein Solution Irradiated with Cells	Fluorescein Irradiated Alone. Cells Added in Dark	Fluorescein Not Irradiated
per cent			
1.0	P	(H)	—
0.5	P	(H)	—
0.25	P	(H)	—
0.125	H	(H)	—
0.062	H	(H)	—
0.031	H	(H)	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

when the dye is irradiated together with the cells than when irradiated separately is a generalization to which many exceptions occur, due chiefly to the complicating factor of fixation which will be considered in a later paper. That hemolysis may proceed farther in the former case than in the latter, in conditions where fixation is not a complicating

TABLE VI

Comparison of Hemolytic Activity of Eosine Irradiated With and Without Blood Cells

Solutions exposed to sunligh tfor one hour and 30 minutes. All solutions contain sodium phosphate buffer, pH 6.9, isosmotic with 0.15 M NaCl. Observations made after 7 hours in dark following irradiation. Symbols as in the preceding tables.

Concentration of Eosine	Eosine Solution Irradiated with Cells	Eosine Irradiated Alone. Cells Added in Dark	Eosine Not Irradiated
per cent			
1.0	H	H	H
0.5	(H)	H	(H)
0.25	H	H	—
0.125	H	H	—
0.062	H	H	—
0.031	H	H	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

factor, seems justified by all the writer's observations on red blood cells under the two conditions. It would be, of course, absurd to attempt an exact quantitative comparison between the results in Table IV and those in Tables V, VI, and VII, since we do not know in the case of the blood cells, what substances may be subject to oxidation, or what their oxidation-reduction potentials may be.

The action of non-irradiated dyes, previously mentioned, is in all probability not an oxidative process, since oxidation of iodide ion by these dyes does not take place in the dark. Whatever the nature of this process, however, when hemolysis occurs after irradiation in a concentration of dye which does not produce hemolysis when not irradiated, we are justified in the assumption that the changes bringing about hemolysis may be oxidative, since we know that the oxidizing power of the dye solution has been increased by irradiation.

TABLE VII

Comparison of Hemolytic Activity of Erythrosine Irradiated With and Without Blood Cells

Solutions exposed to sunlight for one hour. All solutions contain sodium phosphate buffer, pH 7.0, isosmotic with 0.15 M NaCl. Observations made after 6 hours and 20 minutes in dark following irradiation. Symbols as in preceding tables.

Concentration of Erythrosine	Erythrosine Solution Irradiated with Cells	Erythrosine Irradiated Alone. Cells Added in Dark	Erythrosine Not Irradiated
per cent			
1.0	H	H	H
0.5	H	H	H
0.25	H	H	H
0.125	H	H	H
0.062	H	H	H
0.031	H	H	—
0.015	H	(H)	—
0.007	H	—	—
0.004	H	—	—
0.002	H	—	—
0.0	—	—	—

DISCUSSION

Further evidence that oxidation is an important factor in photodynamic processes is not lacking. Oxygen is known to be necessary for a number of photodynamic effects (Straub, 1904*a*; Jodlbauer and Tappeiner, 1905), since they do not take place in its absence. Specifically as regards hemolysis, Hasselbach (1909) found that hemolysis by light and certain photodynamic substances, including eosine and erythrosine, did not take place in a vacuum, and Schmidt and Norman (1922) found that hemolysis by eosine and sunlight did not occur in hydrogen. Sacharoff and Sachs (1905) showed that the presence of the reducing substance *sodium sulfate* may prevent hemolysis by irradiated erythrosine. Noack (1920) showed that a number of inorganic reducing agents may inhibit photodynamic effects, and Schmidt and Norman (1922) found that a number of readily oxidizable organic and inorganic substances will prevent hemolysis by eosine and light. Noack (1920) has also shown quite definitely that certain plant pigments can be oxidized by various photodynamic substances and light, and gives evidence that these phenomena involve the formation of intermediate peroxides.

Conclusions

The demonstration of the formation of an intermediate substance in the process of photodynamic hemolysis by fluorescein dyes offers quite conclusive evidence against the sensitization theory of Tappeiner and other theories which assume that photodynamic substance and substrate must be irradiated together. The demonstration that a definite increase in the oxidizing power of solutions of these dyes is brought about by irradiation, together with the accumulation of other evidence pointing toward an oxidative process, makes it necessary to consider the oxidation of cell constituents as a probable underlying factor in photodynamic hemolysis. Likewise, such oxidations must be considered as a possible factor in all photodynamic processes.

Summary

1. Hemolysis may be produced by previously irradiated fluorescein, eosine and erythrosine.

2. Similarly, previously irradiated fluorescein, eosine and erythrosine oxidize iodide ion.

3. These findings render untenable the sensitization theory of Tappeiner and other theories which necessitate the simultaneous action of light and the photodynamic substance, while supporting Straub's theory of direct oxidation of cell constituents.

4. Oxidation must be considered as a probable underlying cause in photodynamic hemolysis and all other photodynamic phenomena.

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THE EQUILIBRIUM OF OXYGEN WITH THE HEMOCYANIN OF *LIMULUS POLYPHEMUS* DETERMINED BY A SPECTROPHOTOMETRIC METHOD

ALFRED C. REDFIELD

(From the Department of Physiology, Harvard Medical School, Boston, and the Marine Biological Laboratory, Woods Hole)

The respiratory proteins, including hemoglobin, hemocyanin, chlorocrurin and hemerythryn, are unique in combining with and dissociating from oxygen at pressures which fit them for the physiological transportation of this gas. The factors which determine the condition of equilibrium between oxygen and the pigment are of interest not only because of the evident physiological relationship between the characteristics of the oxygen dissociation curves of the blood of various organisms and the pressures of oxygen in the environment, but because of the interesting physico-chemical problem which the phenomena present. The hemocyanins appear to possess certain advantages for the study of these problems. Not only do these proteins exist naturally in solution in the blood so that the complications which arise from dealing with corpuscles are avoided, but they are relatively stable compounds which lend themselves without difficulty to purification and preservation. The hemocyanins of different species appear, in addition, to exhibit very considerable differences in their physical and chemical properties; and consequently, one has the advantage in their study of being able to resort to the comparative method in testing generalizations. Finally, from the technical point of view, the hemocyanins which are essentially colorless when reduced become strongly colored in the oxygenated state and consequently lend themselves to the employment of colorimetric methods for the determination of the degree of oxygenation of the solutions.

The present paper contains an account of a spectrophotometric method for the determination of the degree of oxygenation of hemocyanin solutions. The method is applied to an examination of the equilibrium between oxygen and a purified salt-free preparation of the hemocyanin of the horse-shoe crab, *Limulus polyphemus*, at different hydrogen ion concentrations.

The Spectrophotometric Method

The color of hemocyanin solutions has been taken as an indication of the degree of oxygenation of the protein and used as the basis for constructing oxygen dissociation curves by Pantin and Hogben (1925) and Redfield and Hurd (1925). The method has an advantage over the usual methods of gas analysis in that it requires no correction for the oxygen dissolved in the solution—a correction which is relatively large in comparison to the oxygen content in the case of hemocyanin solutions and which is difficult to determine in a satisfactory manner. When submitted to the proper controls, the method has the advantage that it measures oxyhemocyanin directly by the employment of the spectrophotometer to determine the absorption of monochromatic light of suitable wave-length. Measurements can be made with an ease and accuracy not obtained in the available methods of gas analysis when applied to hemocyanin solutions.

The study of the absorption of light by hemocyanin solutions (Redfield, 1930) affords the essential basis for the employment of the spectrophotometric method. It was shown that the absorption spectra may be analyzed into two components. One is that due to the scattering of light by the solution, the other is that attributable to the true absorption by the chromatic group formed when oxygen unites with the hemocyanin molecule to form oxyhemocyanin. In addition, with the blood of certain animals, the presence of other coloring matters must be taken into account. The component due to the scattering of light is variable depending upon the composition of the solution. It may, however, be readily determined by the study of reduced solutions. The component due to true absorption by the chromatic group was found to be a constant, characteristic of the amount of oxyhemocyanin present, and to vary little if at all with changes in the solution. The presence of other pigments in the blood does not offer complications to the spectrophotometric determination of the absorption of light by the chromatic group, provided these pigments do not undergo change in color with oxygenation; and their influence upon the measurements may be largely avoided by selecting for measurement wave-lengths which are little absorbed by these pigments.

In order to confirm the assumption that the color of a hemocyanin solution is an index of the quantity of oxygen combined with the protein (a supposition heretofore entirely unsupported by exact experiment), the degree of oxygenation of the serum of the horse-shoe crab, *Limulus polyphemus,* was determined simultaneously by the colorimetric method and with the Van Slyke blood-gas analyzer, at a series of oxygen pressures insufficient to produce complete saturation. To 90 cc. of fresh serum, 10 cc. of 0.05 NaOH were added. The pH value of this solution was pH 8.3; the oxygen content was 1.4 volumes per cent when equilibrated with air. Specimens of 10 cc. of serum were equilibrated with air in tonometers evacuated to varying degrees, after the method described by Pantin and Hogben (1925). The tonometers consisted of 250 cc. cylindrical vessels provided with a small test tube sealed on at one end. The other end was closed with a rubber stopper provided with a two-way glass stopcock. When equilibration was complete, the hemocyanin was run down into the small test tube and its color compared with a series of standards made up by diluting the original serum as described by Pantin and Hogben. The tonometers were connected with a reservoir of hydrogen and this gas was allowed to flow in until the pressure was raised to that of the atmosphere. The sample was now withdrawn from the tonometer into a pipette and transferred to a Van Slyke blood-gas analyzer, with which its oxygen content was measured. In estimating the oxygen dissolved in the samples, the solubility coefficient was taken to be 0.0235 (Redfield, Coolidge and Montgomery, 1928), a value closely checked by direct measurements on this specimen of serum.

TABLE I

Comparison of Colorimetric and Gasometric Determination of Degree of Saturation of Limulus Serum with Oxygen

O_2 Pressure	O_2 Content	O_2 Dissolved	O_2 Combined	Saturation	Color
mm. Hg	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	*per cent*
3.1	0.082	0.010	0.072	7.8	10
4.5	0.152	0.014	0.138	15.0	20
5.3	0.235	0.016	0.219	23.8	25
10.8	0.399	0.034	0.365	39.7	40
13.8	0.484	0.043	0.441	48.0	50
15.9	0.548	0.049	0.499	54.4	55
21.7	0.653	0.067	0.586	63.8	65
34.4	0.953	0.106	0.847	92.0	90
155.0	1.400	0.480	0.920	100.0	100

The results of this experiment are recorded in Table I. It may be seen that the degree of saturation of the solution as estimated from its color agrees closely with that determined from the direct measurement of the oxygen combined with the hemocyanin. The use of the spectrophotometer would markedly improve the precision of the colorimetric estimations in this experiment. The errors inherent in the gasometric measurements are, however, so large that the significance of the comparison would not be increased by the further refinement of this part of the experiment.

While the foregoing affords practical demonstration of the utility of colorimetric methods for determining the oxygenation of hemocyanin solutions, confidence in the more precise measurements obtained with the spectrophotometer must be based upon the theoretical adequacy of the procedure.

The analysis of the absorption of light by hemocyanin solutions indicated that, for any wave-length of light

$$E_o = E_x + E_r, \tag{1}$$

where E_o is the extinction coefficient of the oxygenated solution, E_x is the extinction coefficient characterizing the absorption of light by the chromatic groups in the oxygenated solution, and E_r is the extinction coefficient of the reduced solution. It was shown that Beer's law applies to hemocyanin solutions in both the oxygenated and reduced condition. One may consequently write:

$$E_o = cK_o,$$

$$E_r = cK_r,$$

$$E_x = cK_x,$$

where c is the concentration of hemocyanin and K_o, K_r, and K_x are the extinction coefficients at unit concentration. It follows that

$$E_o = cK_x + cK_r. \tag{2}$$

If E_y be the extinction coefficient characteristic of a mixture of oxygenated and reduced hemocyanin in which the concentration of oxygenated hemocyanin is yc and that of reduced hemocyanin is $(1 - y)c$,

$$E_y = y(cK_x + cK_r) + (1 - y)cK_r, \tag{3}$$

$$E_y = ycK_x + cK_r. \tag{4}$$

Substituting E_r for cK_r in equations (2) and (4), dividing and rearranging,

$$y = \frac{E_y - E_r}{E_o - E_r}\,. \tag{5}$$

This result is obtained without any explicit assumption regarding the cause for the absorption of light measured by E_r, and the equation may consequently be applied in determining the degree of saturation of solutions in which other pigments as well as scattering effects are responsible for the value of this term. It may also be derived by means of a slightly different argument for cases such as that exhibited by hemoglobin solutions, in which the prosthetic group absorbs considerable but different quantities of light in the oxygenated and reduced condition.

It is assumed in the foregoing that an incompletely saturated solution of hemocyanin is a mixture of completely reduced and completely oxygenated elements. No account is taken of the possibility that incompletely oxygenated molecules may occur which possess absorption spectra different from that of the completely oxygenated solution.

This possibility cannot be ignored in view of the success which theories of intermediate degrees of oxygenation have met in explaining the characteristics of the oxygen dissociation curves of hemoglobin (Adair, 1925; Ferry and Green, 1929), even though the experiments of Conant and McGrew (1930) failed to demonstrate the existence of such intermediate compounds. The high molecular weights reported for hemocyanins (Svedberg and Chirnoaga, 1928; Svedberg and Heyroth, 1929) definitely indicate that many oxygen molecules may combine with each hemocyanin molecule. If the chromatic group undergoes intermediate degrees of oxygenation, this will affect the foregoing deduction only in so far as the spectrum of the partially oxygenated chromatic group differs from that of the completely oxygenated chromatic group. The spectrum of the chromatic group in a partially saturated solution has consequently been determined and compared with that of the completely oxygenated solution. The result is tabulated in Table II. The ratio of the extinction coefficients of the chromatic group of fully and partially oxygenated solutions is practically the same at all wave lengths, which indicates that the partially saturated solution does not contain intermediate compounds which differ in their spectral characteristics from the fully oxygenated solution.

TABLE II

Comparison of Spectrum of Fully Oxygenated and Partially Oxygenated Hemocyanin of Limulus. Concentration, .0258 grams per cc.; length of tube, 3.3 cm.; pH = 7.43; "Salt Free."

Wave-Length	Equilibrated with 726 mm. O_2	Equilibrated with 2.6 mm. O_2	Equilibrated with H_2	Oxygenated Chromatic Group	Partially Oxygenated Chromatic Group	y
$m\mu$	E_o	E_y	E_r	$E_o - E_r$	$E_y - E_r$	$\frac{E_y - E_r}{E_o - E_r}$
460	0.157	0.100	0.034	0.123	0.066	.536
480	0.136	0.086	0.034	0.102	0.052	.510
500	0.151	0.094	0.030	0.121	0.064	.529
520	0.199	0.120	0.028	0.171	0.092	.538
540	0.253	0.150	0.026	0.227	0.124	.546
560	0.288	0.170	0.026	0.262	0.144	.550
580	0.303	0.178	0.027	0.276	0.151	.547
600	0.302	0.172	0.025	0.277	0.147	.530
620	0.285	0.168	0.025	0.260	0.143	.550
640	0.265	0.155	0.024	0.241	0.131	.544
660	0.244	0.143	0.028	0.216	0.115	.532
680	0.216	0.127	0.028	0.188	0.099	.528
700	0.200	0.117	0.022	0.178	0.095	.530

The Determination of the Oxygen Dissociation Curve

Measurement of Degree of Oxygenation.—The extinction coefficients of the solutions were measured with the aid of a König-Martens spectrophotometer. They are given by equations of the type

$$E_o = \frac{2(\log \tan a_o - \log \tan a_1)}{d}, \tag{6}$$

where a_o is the angle of the analyzing Nicol prism when oxyhemocyanin is measured; a_1 is the angle when the absorption of light by the solvent is determined; and d is the length of the absorbing column. Similarly, expressing the angular reading characteristic of an incompletely saturated solution as a_y and that of the reduced solution as a_r, E_y and E_r are obtained. Substituting in (5) the degree of saturation, y is given by the expression

$$y = \frac{\frac{2}{d}\log \tan a_y - \frac{2}{d}\log \tan a_r}{\frac{2}{d}\log \tan a_o - \frac{2}{d}\log \tan a_r}. \tag{7}$$

Since the corrections for the absorption of light by the solvent cancel out, they need not be measured. In practice the greatest accuracy is obtained at wave-lengths giving the greatest difference between the values of E_0 and E_r. In the case of hemocyanin solutions, this occurs in the yellow region of the spectrum. This fact is fortunate in that this is the region in which readings can be made with the greatest accuracy. It is also a practical advantage that the scattering of light is small at these wave lengths and variations due to changes in the physical conditions of the solution are consequently minimal. In preparing solutions, the greatest precision is obtained by adjusting the concentration and the length of the absorbing chamber so that the angle a_0 is as large as is compatible with precise measurement, that is, about 75°.

Equilibration with Oxygen.—In order to equilibrate the solutions with oxygen of known pressure, tonometers such as those illustrated in Fig. 1 were employed. These consist of cylindrical bottles of Pyrex glass, having a capacity of 250 cc., to one end of which is sealed a T-tube having an internal diameter of approximately one centimeter. The ends of the T are ground parallel to one another and are closed with flat glass disks sealed on with DeKhotinsky cement. The mouth of the bottle is closed with a rubber stopper in which a two-way glass stopcock is inserted. About five cc. of the solution to be measured is placed in the tonometer, which is then evacuated and refilled with a gas mixture containing a convenient proportion of oxygen. In the experiments described in this paper, nitrogen containing 2 to 5 per cent of oxygen has been convenient; for other solutions, air will serve or pure oxygen may be necessary. The tonometer is now evacuated to some definite pressure and the solution equilibrated with the gas at this pressure, rotating the tonometer in a horizontal position for fifteen minutes in a water bath at constant temperature. The tonometer is next returned to a vertical position and evacuated and filled again with the gas mixture and pumped out to the same reduced pressure. The solution is further equilibrated for twenty-five minutes and then, without removal from the water bath, is turned into the vertical position and connected with the manometer after setting the pressure in the system to that expected to obtain in the tonometer. The passage connecting the tonometer and manometer with the pump and reservoir is now closed. The stopcock leading into the tonometer is opened, and the pressure obtaining in the tonometer is carefully measured and recorded. The stopcock of the tonometer is closed again and disconnected from the pump. The tonometer is carefully dried and placed in an air-chamber inserted into the water bath where it is kept for a period of approximately one hour in order that the small particles of denatured protein which almost invariably form during the process of evacuation and equilibration may

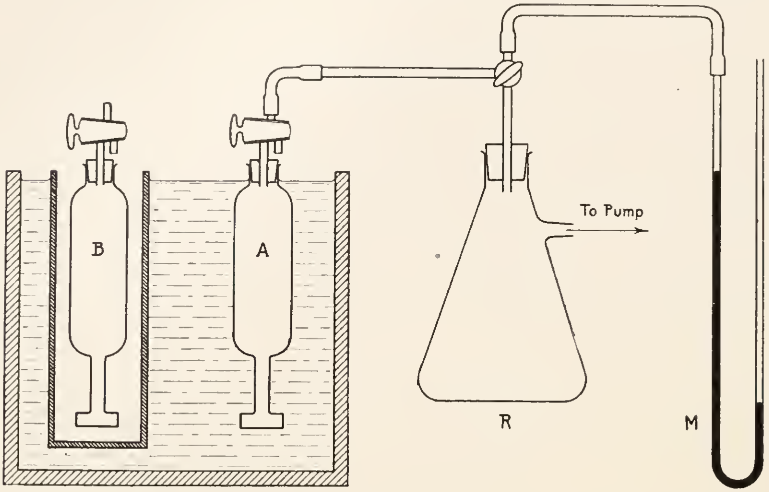

Fig. 1. Arrangement for adjusting pressure of gas in tonometers. *A*, tonometer in position for measuring pressure after equilibration. *B*, tonometer in thermostat during settling of sediment. *M*, mercury manometer. *R*, reservoir which serves to slow rate of evacuation of tonometers.

settle out. Following this, the absorption of light by the hemocyanin solution is measured by placing the tonometer with the T-tube in the path of one of the beams of the spectrophotometer. If the solutions are not clear at the time when the measurements are made, the results should be rejected.

The partial pressure of oxygen in the tonometer, p, is given by the expression

$$p = (B - P - aq)\, f,$$

where B is the barometric pressure, P is the pressure in the tonometer recorded at the end of equilibration, aq is the tension of aqueous vapor at the temperature of the water bath, and f is the fraction of oxygen in the gas mixture.

To obtain complete oxygenation, the tonometer is simply evacuated and filled with pure oxygen gas prior to equilibration. To obtain complete reduction is difficult under those circumstances in which the affinity of hemocyanin for oxygen is great. We have not found the employment of chemical reducing agents satisfactory, as certain of these tend to influence the color of the solution and others must be employed in such concentrations that they may affect the scattering of light on which the absorption by the reduced solution depends. The most satisfactory procedure is to employ hydrogen to wash out the tonometer after the oxygen is freed from the hemocyanin under low pressure. The solutions are accordingly evacuated, equilibrated for twenty minutes, filled with hydrogen, re-evacuated, again equilibrated, allowed to settle, and then measured. Further repetition of the process does not lead to lower readings, although it is doubtful whether, under certain circumstances, this process removes the last traces of oxyhemocyanin. The reason for this is that, in the process of evacuation and equilibration, small quantities of denatured material are formed which fail to settle out completely when the solutions are allowed to stand. The formation of precipitates of this sort, which goes on more readily in the reduced solutions, constitutes the principal limit to the precision of the method. We have recently constructed tonometers which can be placed in the cups of a large centrifuge and which make it possible to remove these troublesome precipitates. Such tonometers have not been employed in the experiments described in this paper.

The Preparation of the Hemocyanin Solutions.—The hemocyanin employed in the present investigation was prepared from material obtained during the summer of 1928. It was preserved in the precipitated state by adding 350 grams of ammonium sulphate to each liter of serum. The material was purified some months later by repeated salting out followed by dialysis, against dilute sodium hydroxide, as described by Redfield, Coolidge and Shotts (1928). Three preparations were obtained, having the following characteristics: Specimen 18 *A*, dry weight 0.1031 gram per cc., combined base 19.4×10^{-5} mols per gram; Specimen 18 *B*, dry weight 0.1255 gram per cc., copper 0.0208 milligram per cc. or 0.168 gram per 100 grams dry substance, combined base 19.1×10^{-5} mols per gram; Specimen 18 *C*, dry weight 0.097 gram per cc., copper 0.16 milligram per cc. or 0.165 gram per 100 grams dry weight, combined base 21.6×10^{-5} mols per gram. These solutions were preserved with toluene at a low temperature. The day before measurements were to be made, they were further diluted by the addition of distilled water containing amounts of hydrochloric acid or sodium hydroxide appropriate to secure the desired hydrogen ion activity and to reduce the hemocyanin to a concentration favorable for the measurements; that is, to about 2.5 per cent. After standing all night, the solutions were filtered and then employed for the determination of the oxygen dissociation curves. A portion of the solution was also reduced by equilibration with hydrogen and used for the determination of the hydrogen ion concentration by means of the hydrogen electrode.[1]

Measurements were made upon solutions at several hydrogen ion activities between pH 7.4 and pH 10.4, and upon a solution at pH 4.5. At hydrogen ion activities intermediate between pH 4.5 and about pH 6.8, *Limulus* hemocyanin is insoluble in distilled water, and solutions of sufficient clarity cannot be obtained. At reactions more acid than pH 4.5, a colorless modification of *Limulus* hemocyanin is formed (Redfield and Mason, 1928). The characteristics of the oxygen dissociation curve at these hydrogen ion activities will be dealt with in a subsequent paper.[2]

DATA ON OXYGEN DISSOCIATION

The results of the series of measurements which have been made upon purified solutions of *Limulus* hemocyanin are recorded in Table III. The first column contains a description of the material employed in each case; the second column, the partial pressure of oxygen in the tonometer at the completion of equilibration; the third column, the value of the extinction coefficient of the solution ($2/d \log \tan a_y$), as measured with the spectrophotometer, employing light of the wave-length

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[1] In the case of the two solutions prepared from Specimen 18 *C*, the pH value was somewhat less than that to be expected from the amount of NaOH added, as judged from the titration curve published by Redfield, Humphreys and Ingalls (1929). In the other solutions the agreement is good.

[2] I am indebted to Miss Elizabeth Ingalls for technical assistance in conducting the experiments and for preparing the hemocyanin solutions employed.

590 mμ. This measurement is not corrected for absorption by the solvent. The fourth column records the extinction coefficient of the oxygenated chromatic groups, $2/d(\log \tan a_y - \log \tan a_r)$; the fifth column, the value of y as defined in equation (7). The equilibration was carried out in a water bath at 25° C. The length of the T-tube of the tonometer in which the absorption of light was measured, d, was usually 3.3 centimeters. In the case of a few measurements, tubes were employed which differed slightly from this length (3.15 to 3.60 cm.).

THEORY OF OXYGEN EQUILIBRIUM

In oxyhemocyanin, one molecule of oxygen is bound by a quantity of hemocyanin containing two atoms of copper. The reversible reaction may consequently be indicated by the equation

$$RCu_{2n}O_{2n} = RCu_{2n} + nO_2,$$

where n represents the number of mols of oxygen bound by each mol of hemocyanin. In treating the equilibrium according to the mass law, as was done by Hüfner (1901) and later by Hill (1910), in the case of hemoglobin, the result is

$$\frac{(RCu_{2n}O_{2n})}{(RCu_{2n})(O_2)^n} = k, \tag{8}$$

in which k is the equilibrium constant of the reaction. If y is the fraction of hemocyanin in the oxygenated condition, $1 - y$ is the reduced fraction and, putting p, the partial pressure of oxygen in mm. of mercury, in place of the oxygen concentration, equation (8) may be written

$$\frac{y}{1 - y} = Kp^n \tag{9}$$

or,

$$\log\left(\frac{y}{1 - y}\right) = \log K + n \log p. \tag{10}$$

In this form the equation is convenient for graphical solution for n and K.

In Fig. 2 is reproduced the data recorded in Table I, arranged in the form indicated by equation (10). The lines drawn through the points in each case are straight lines indicating the linear relationship demanded by the equation. The slope of the lines drawn through the points, determining the value of n, is 1.0. The values of K corresponding to the positions of the lines drawn in Fig. 2 are indicated in Table III. Employing these values of K and taking n as equal to 1.0 in each case, the values of y may be calculated and are indicated in column 6 of

Table III for comparison with the observed values. It appears that the theoretical treatment from which equation (10) is derived is adequate to account for the shape of the oxygen dissociation curve at least

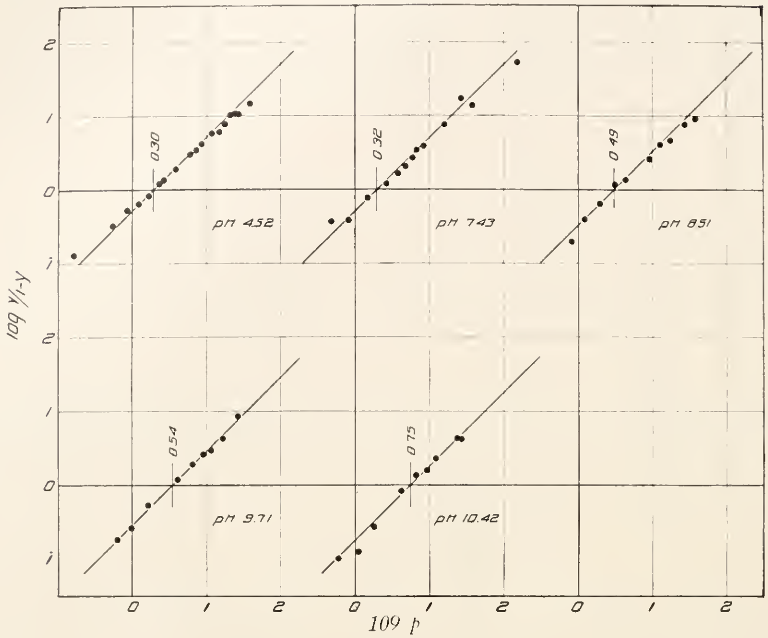

FIG. 2. Logarithmic plot of data of oxygen dissociation curve of hemocyanin of *Limulus polyphemus* at various pH values. Temperature, 25° C.; y, fraction of hemocyanin in oxygenated condition; p, oxygen pressure in mm. Hg.

as a first approximation, and to provide a single series of constants to define the effect of hydrogen ion activity upon the equilibrium.[3] Careful scrutiny of the data in Table III reveals a tendency for the low values of y to be slightly greater than the calculated values and high values to be slightly less than the theoretical. In order to make vivid the adequacy of the theory for treating the entire set of observations, in Fig. 3 the values of y obtained at each pH value are plotted against Kp in the usual form of the oxygen dissociation curve, and a line corresponding to the theoretical treatment is drawn through the points, n again being taken as 1.0.

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[3] It should be emphasized that the pH values are determined on reduced solutions. No account has been taken of possible change in pH with oxygenation. According to Redfield, Humphreys and Ingalls (1929), the effect may be expected to be small.

TABLE III

Data of Oxygen Dissociation Curves of Limulus Hemocyanin. Temperature, 25° C.; Wave-length, 590 mμ.

Description	p	$\frac{2}{d}\log\tan a_y$	$\frac{2}{d}\log\frac{\tan a_y}{\tan a_r}$	y	y
	mm. Hg			*(observed)*	*(calculated)*
Specimen 18 *A*	0	0.034	0	0	0
	0.16	0.068	0.034	0.117	0.074
Concentration:	0.56	0.106	0.072	0.248	0.218
0.0258 grams per	0.80	0.133	0.099	0.342	0.286
cc.	1.24	0.149	0.115	0.397	0.382
Combined acid:	1.65	0.166	0.132	0.455	0.452
20×10^{-5} mols	2.34	0.194	0.160	0.552	0.540
per gram	2.74	0.204	0.170	0.586	0.578
pH 4.52	3.92	0.225	0.191	0.659	0.662
	6.20	0.252	0.218	0.752	0.757
$K = 0.500$	7.28	0.259	0.225	0.776	0.784
	8.65	0.269	0.235	0.810	0.812
	11.9	0.281	0.247	0.852	0.856
	14.8	0.283	0.249	0.859	0.881
	17.6	0.291	0.257	0.886	0.898
	20.6	0.298	0.264	0.910	0.913
	24.5	0.299	0.265	0.914	0.924
	27.4	0.299	0.265	0.914	0.933
	39.0	0.305	0.271	0.935	0.952
	744	0.324	0.290	1.00	1.00
Specimen 18 *A*	0	0.034	0	0	0
	0.39	0.092	0.058	(0.265)	0.171
Concentration:	0.80	0.119	0.085	0.305	0.276
0.0258 grams per	1.49	0.158	0.124	0.441	0.415
cc.	2.61	0.188	0.154	0.548	0.554
Combined base:	3.74	0.207	0.173	0.616	0.640
19×10^{-5} mols	4.78	0.223	0.189	0.672	0.695
per gram	5.96	0.239	0.205	0.730	0.739
pH 7.43	6.83	0.253	0.219	0.780	0.766
	8.38	0.258	0.224	0.797	0.800
$K = 0.476$	16.6	0.282	0.248	0.882	0.888
	26.4	0.300	0.266	0.946	0.927
	37.7	0.297	0.263	0.936	0.948
	152	0.310	0.276	0.982	0.987
	740	0.315	0.281	1.00	1.00

TABLE III (*continued*)

Data of Oxygen Dissociation Curves of Limulus Hemocyanin. Temperature, 25° C.; Wave-length, 590 mμ.

Description	p	$\frac{2}{d}\log\tan a_y$	$\frac{2}{d}\log\frac{\tan a_y}{\tan a_r}$	y	y
	mm. Hg			*(observed)*	*(calculated)*
Specimen 18 *B*	0	0.046	0	0	0
	0.84	0.078	0.032	0.166	0.213
Concentration:	1.22	0.103	0.057	0.295	0.282
0.0208 grams per	1.98	0.123	0.077	0.399	0.390
cc.	3.15	0.150	0.104	0.539	0.504
Combined base:	4.40	0.158	0.112	0.580	0.587
39×10^{-5} mols	9.30	0.185	0.139	0.720	0.750
per gram	12.80	0.201	0.155	0.803	0.805
pH 8.51	17.7	0.204	0.158	0.818	0.852
	28.1	0.217	0.171	0.886	0.900
$K = 0.322$	39.0	0.219	0.173	0.902	0.927
	751	0.239	0.193	1.00	1.00
Specimen 18 *C*	0	0.047	0	0	0
	0.63	0.082	0.035	0.155	0.177
Concentration:	0.97	0.093	0.046	0.204	0.217
0.0242 grams per	1.64	0.128	0.081	0.358	0.319
cc.	4.12	0.167	0.120	0.531	0.541
Combined base:	6.38	0.197	0.150	0.654	0.646
63×10^{-5} mols	8.94	0.209	0.162	0.717	0.719
per gram	11.5	0.216	0.169	0.748	0.767
pH 9.71	16.8	0.230	0.183	0.810	0.828
	21.8	0.228	0.181	(0.801)	0.862
$K = 0.286$	26.7	0.250	0.203	0.898	0.885
	724	0.273	0.226	1.00	1.00
Specimen 18 *C*	0	0.066	0	0	0
	0.61	0.084	0.018	0.092	0.098
Concentration:	1.10	0.088	0.022	0.112	0.164
0.0242 grams per	1.79	0.124	0.058	0.296	0.242
cc.	4.15	0.155	0.089	0.454	0.426
Combined base:	6.53	0.177	0.111	0.566	0.538
77×10^{-5} mols	9.04	0.183	0.117	0.597	0.618
per gram	12.2	0.202	0.136	0.694	0.686
pH 10.42	17.1	0.242	0.176	(0.898)	0.754
	22.6	0.225	0.159	0.812	0.802
$K = 0.178$	27.2	0.224	0.158	0.806	0.830
	751	0.262	0.196	1.00	1.00

DISCUSSION

In the forty years since Hüfner suggested the application of the mass law to the equilibrium between oxygen and hemoglobin, numerous investigations have indicated that equations similar to those employed in the present treatment are more or less adequate to describe the data in hemoglobin solutions free of electrolytes (Barcroft, 1928). Uncertainty has sometimes accompanied the results of such investigations because of the instability of purified hemoglobin solutions (Ferry, 1924; Hecht, Morgan and Forbes cited by Barcroft, 1928). In the presence of electrolytes and in blood, the dissociation curves of hemoglobin invariably have a sigmoid shape, requiring some additional assumptions for their explanation.

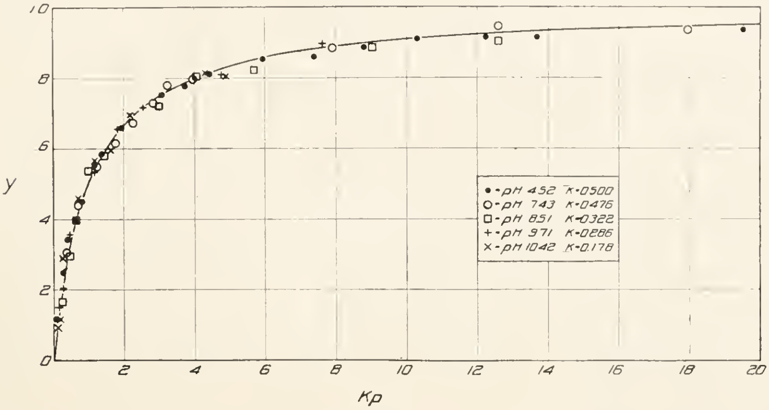

FIG. 3. Data of oxygen dissociation curves of hemocyanin of *Limulus polyphemus* plotted to show the similarity of shape at various pH values. y is fraction of hemocyanin present as oxyhemocyanin; p is oxygen pressure in mm. Hg; temperature, 25° C. The curve corresponds to equation (9) when $K = 1$, $n = 1$.

In the case of hemocyanin solutions, Stedman and Stedman (1928) found that the respiratory pigment of the snail, *Helix pomatia*, combines oxygen in accordance with the mass law, as expressed in equation (8), n being taken to be 1.0. The hemocyanin of the crustacea, *Homarus vulgaris* and *Cancer pagurus*, according to these investigators (1926 *a*, 1926 *b*) is characterized by oxygen dissociation curves of a more complex nature when examined in dialyzed solution. The present investigation of *Limulus* hemocyanin indicates that this substance, when in "salt free" solutions, resembles the hemocyanin of *Helix* in its conformity to the mass law.

Stedman and Stedman, in discussing their observations on *Helix* hemocyanin, conclude from the fact that the value of n is 1.0, that this hemocyanin is dispersed in solution in such a way that each hemocyanin molecule unites with but a single oxygen molecule. It is tempting to draw the same conclusion with regard to *Limulus* hemocyanin, for the investigations of Redfield, Coolidge and Shotts (1928) indicated that the probable molecular weight of this protein is 73,400 and that each molecule contains two atoms of copper. The measurements of Redfield, Coolidge and Montgomery (1928) demonstrate further that such a hemocyanin molecule would bind but a single oxygen molecule. The value of n established in this investigation follows as a prediction from these considerations. It must be recalled, however, that Svedberg and Heyroth (1929) obtained much larger values for the molecular weight of *Limulus* hemocyanin by the employment of the ultra-centrifugal method. In view of the uncertainty regarding the size of the hemocyanin molecule, reserve is required in interpreting the data of the oxygen equilibrium. If one goes back to the kinetic basis of the mass law equation (8), it may be noted that the fundamental assumptions concern the probability of the union of an oxygen molecule with the respiratory protein and the probability of the dissociation of such a union. Where expressions arise giving values of n greater than 1.0, or more complicated equations, it is through the assumption that some relation exists between the combination of oxygen by contiguous groups; either that they unite with oxygen simultaneously as pairs or larger groups, or that they combine in successive steps so that one cannot react until after others have done so. All that can safely be concluded from a demonstration that hemocyanin unites with oxygen as though it were dispersed in molecules each combining with but a single oxygen molecule is that it behaves *as though* this were the case. That is to say, the oxygen dissociation curve is such as would be obtained if the various oxygen binding groups reacted independently of one another so that the oxygenation of any one did not influence the probability of oxygenation or reduction of any other. That this may be the case in a molecule containing a number of oxygen-binding groups does not seem altogether impossible when it is recalled that the molecular weight of such a molecule would be 73,400 times the number of groups. It should be recalled that in combining with acid, the molecule of *Limulus* hemocyanin, which binds at least 117 equivalents of acid, behaves as though each acid-binding group reacted independently of every other (Redfield and Mason, 1928). In this regard the behavior of this protein is not exceptional.

The measurements recorded in Table III make it clear that as alkali is added to solutions of purified *Limulus* hemocyanin the equilibrium constant of oxygenation decreases progressively, indicating that greater pressures of oxygen are required to produce any given degree of oxygenation. No suggestive relationship is apparent between the values of K, and either the quantity of alkali added or the hydrogen ion activity of the solution. It is noteworthy that the phenomena exhibited by these purified solutions of hemocyanin differ markedly from those obtaining in the native serum of *Limulus*. As pointed out briefly by Hogben and Pinhey (1927) an extensive series of measurements on the oxygen dissociation curves of *Limulus* serum (which we have not published) demonstrate that at pH values up to about 8.3 the oxygen pressure requisite to produce a given degree of oxygenation increases. At higher pH values these pressures decrease again, much as is the case with *Helix aspersa* blood (Hogben and Pinhey, 1926).

In their investigation of the dialyzed hemocyanin of *Helix pomatia*, Stedman and Stedman (1928) report that no detectable change in the curve with change in pH was observed. Experiments now in progress with the purified hemocyanin of *Busycon canaliculatum* agree closely with the findings in the case of *Limulus*, indicating a definite decrease in the value of K with diminishing hydrogen ion activity. Inasmuch as the results with *Helix* are otherwise very similar to those obtained with *Limulus* and *Busycon* hemocyanin, we have reëxamined the Stedmans' data and find evidence suggesting that, with this material, there may be a small effect of hydrogen ion concentration upon the value of K. In a set of curves defined by equation (9), differing only in the value of K and where n equals 1.0, the greatest differences in y obtain between degrees of saturation of 0.40 and 0.80. It is in this range that differences in the curves may be most readily detected. We have consequently evaluated K on the basis of their data selected between these degrees of saturation. The results are presented in Table IV. The average value of K for the data selected is 0.250. Of the nine measurements made on solutions more alkaline than pH 7, the mean value is 0.217 and the highest value is 0.236. Of the ten measurements made on solutions more acid than pH 7, the average value is 0.270, and only two values are less than 0.236. This result indicates that a small but definite change in the value of K may occur in the case of *Helix* hemocyanin with change in hydrogen ion concentration, and that the phenomena in this case may not differ qualitatively from that obtaining with *Limulus* and *Busycon*.

TABLE IV

The Equilibrium Constant of Oxygenation of HELIX POMATIA *Hemocyanin at Various pH Values Calculated from the Data of Stedman and Stedman (1928)*

pH	p	Per cent Saturation (corrected)	Per cent Unsaturated	K
	mm. Hg	$100 \times y$	$100 \times (1 - y)$	
4.04	2.89	47.9	52.1	0.318
	8.38	70.3	29.7	0.282
	11.61	72.4	27.6	0.226
4.79	2.74	40.4	59.6	0.247
	6.73	67.9	32.1	0.314
6.25	2.85	46.7	53.3	0.307
	6.45	64.5	35.5	0.282
	11.13	70.8	19.2	0.331
6.35	3.79	47.9	52.1	0.243
	11.30	62.6	37.4	0.148
7.81	2.83	39.8	60.2	0.234
	7.00	57.1	42.9	0.190
	7.96	62.8	37.2	0.212
8.74	2.90	40.6	59.4	0.236
	6.15	59.1	40.9	0.235
	10.04	65.8	34.2	0.191
9.02	2.50	37.0	63.0	0.235
	6.00	55.6	44.4	0.209
	7.51	62.1	37.9	0.218

SUMMARY

A spectrophotometric method for measuring the equilibrium of hemocyanin and oxygen is described.

The oxygen dissociation curves of purified hemocyanin of *Limulus* in the absence of salts and at various hydrogen ion activities are determined.

It is shown that the equilibrium between oxygen and these hemocyanin solutions is defined, as a first approximation, by the mass law on the assumption that the various oxygen-combining groups react independently of one another in their combination with oxygen.

The value of the equilibrium constant of the oxygenation reaction decreases as the pH value increases from 4.5 to 10.4.

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POLOCYTE FORMATION AND THE CLEAVAGE OF THE POLAR BODY IN *LOLIGO* AND *CHÆTOPTERUS*

LEIGH HOADLEY[1]

(From the Department of Zoölogy, Harvard University, and the Marine Biological Laboratory, Woods Hole, Massachusetts)

The polar bodies which arise during the maturation of the eggs of animals may be considered from two points of view. Most attention has in the past been paid to the first, that of their meaning in the maturation of the nucleus, which we will but mention here. These phenomena are similar to those appearing in the maturation of the sperm cells and hence, to generalize, the maturation of the egg cell may be said to parallel that of the sperm as far as the reduction and equation divisions are concerned. In addition there is the second point, that which concerns the function and fate of the polar bodies (aborted ova, Mark 1881). This question has been shown by Conklin to be intimately associated with the history of the ovum and the relation of its maturation phenomena to the penetration of the spermatozoön. In addition, it appears that one must consider the constitution of the polar body itself, for in some cases the protoplasm extruded from the egg includes more of the cytoplasmic regions (qualitatively) than in others. It is evident that this qualitative difference in the division of the egg would be of more significance than the mere quantitative division, though great quantitative discrepancy in the equivalence of the daughter cells would also be of great importance.

Observations have been made during the past summer which confirm in the living egg the observations recorded by Lillie in experiments on *Chætopterus* that the polar body of that form does not include all of the constituent parts of the egg which continues to develop. These results, when compared with some obtained in the study of the fate of the polar bodies in *Loligo,* give a clew as to some of the factors important to the non-developmental capacity of this structure. The polar body of *Loligo* may divide several times as will be described below. In *Chætopterus,* the polar body does not divide and in this connection it should be noted that in its formation, none of the cortical region of the egg is involved so that the polar body represents only endoplasmic cytoplasm and nucleus. This point would seem to be of great impor-

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[1] This investigation was aided by grant from the Milton Fund.

tance as far as the developmental capacity of the gamete is concerned. We will consider the observations on these forms first and return to the consideration of their significance and the relation between them and other data already obtained below.

Loligo pealii

Eggs and sperm of the squid, *Loligo pealii,* may be obtained in large numbers from animals during the month of July at Woods Hole. The mature eggs are found in the body cavity of the females and may best be obtained in an uninseminated condition by opening the animal on the posterior side. The eggs are clear and show a very distinct micropyle as has been described by Watasé (1891). Sperm may be obtained from spermatophores produced in large numbers by the male. Several of the spermatophores may be placed in a small amount of sea-water and then broken or cut so that the sperm flow out into the medium. This fluid may then be used to inseminate the eggs, which should be placed in a small amount of sea-water. Owing to the large size of both the eggs and the sperm and the clarity with which the well-formed micropyle may be seen, the entire course of the sperm through the micropyle and to the egg may be observed. As the sperm penetrates the egg, the polar cap may be seen to elevate, the cytoplasm streaming to that portion of the egg directly under the inner opening of the micropyle where it forms the blastodisc. In the squid, the penetration of the spermatozoön normally takes place before the formation of either of the polar bodies.

After the polar cap is well elevated, the first polar body appears. This is relatively small though actually much larger than in *Chætopterus,* for example. It appears first as a slight elevation on the surface of the egg, which rapidly pinches it off. The polar body maintains its approximate position on the surface of the egg, however, so that after a short interval it serves as a locus in determining the site of the formation of the second polar body. This forms and the first polar body divides. At that time the male pronucleus may be seen in the cytoplasm of the egg as a bright spot near, but not in contact with, the female pronucleus. The two gradually approach and are then lost to view in the living egg. The egg then continues in its development, cleaving, as has been described by Watasé, to form a blastodisc covering first the polar end of the egg and subsequently spreading around the yolk from this point.

If, during the period immediately following the juxtaposition of the two pronuclei, we observe the polar bodies, we find that these may continue their development for a short time. Not only may the first polar body divide, as is the case in many forms, but the second may also divide or the first may show a division in one or both of its daughter-cells. This is not always the case but is found in a large number of the eggs of this form. In Fig. 1 may be seen a number of cases in which there are four or five of the cells produced by the polar bodies attached to the cortical portion of the egg and forming a nest of cells there. When observed both in their division and afterwards, these are evidently not the result of fragmentation of the cytoplasmic portion of the polar body, but are real products of the division of these units. No observations have been made in which more than six cells were counted which resulted from the division of the polar bodies. Eggs with five cells are not rare and those with four are common.

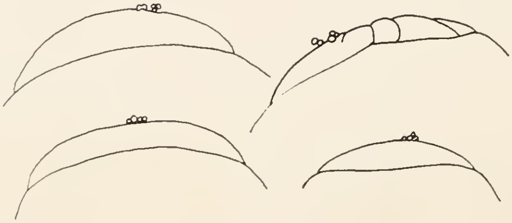
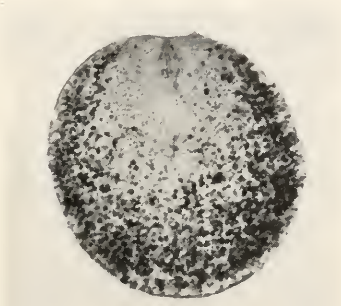


FIG. 1. Sketches of eggs of *Loligo* showing four and five polar bodies attached to the surface.

The next series of observations on the extrusion of the polar bodies in *Loligo* is difficult to make because of certain optical requirements. The formation of the polar bodies was observed with the cardioid condenser. In the use of this, the object observed must be a specific distance from the condenser, which, owing to the great thickness of the squid egg, requires the use of a cover glass as a slide and some manipulation of the material. In the course of a large number of attempts, however, certain of them were successful, so that the following description is based on a number of observations of the phenomenon. As is the case with *Chætopterus,* which will be considered below, the cytoplasmic portion of the egg of *Loligo* is covered with a thin cortical zone. This zone is thinner than in *Chætopterus* but similar in that structurally it differs from the deeper layers. When the site of polar body formation has been determined by the approach of the nucleus and the elevation of the small mound from which it arises, it can be seen that the cortical region is contained in the part elevated. In other words, the portion of the cytoplasmic cap which goes into the polar body is composed of a thin cortical region and deeper endoplasmic portion. In addition, this receives, of course, a share of the maturation nucleus. The second polar body is formed in essentially the same manner. This fact is, I think, of great significance in the consideration of the subsequent cleavage of the polocytes.

Chætopterus pergamentaceus

The discussion and description of the formation of the polar bodies of *Chætopterus* consists, insofar as the description of the living and fixed egg is concerned, merely in a confirmation of the observations of Lillie (1906). In addition, the egg has been studied by means of the cardioid condenser, which enables the investigator to trace the cortical, or as it is called in the above paper, the ectoplasmic portion of the egg, and to distinguish it from the endoplasmic portion during the maturation stages. The observations are entirely in accord with those cited above, but will be described here in order that they may be referred to in the discussion.



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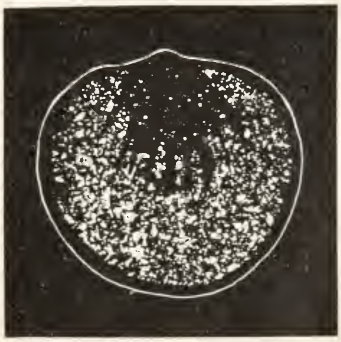

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FIG. 2. Photograph of a section of a *Chætopterus* egg at the mesophase of the first maturation division, showing the "ectoplasmic defect" and the arrangement of adjacent parts.

FIG. 3. Sketch of an egg of *Chætopterus* at the mesophase of the first maturation division as it appears by dark field illumination with the cardioid condenser to show the "ectoplasmic defect" as the polar elevation is forming.

As is well known, the egg of *Chætopterus* is penetrated by the spermatozoön before the formation of the polocytes. Thus activated, the cortical or ectoplasmic portion withdraws from the animal pole of the egg, and the spindle of the first oöcytic division approaches the cell membrane remaining surrounded by the endoplasmic cytoplasm. It becomes attached to the outer zone in the region of the ectoplasmic defect and there forms the first polar body. This has been described by Lillie (*cf.* Fig. 2) and may be seen in living eggs by means of the cardioid condenser (Fig. 3). It is composed entirely of endoplasmic substance without deutoplasm. The polar body must, therefore, represent ground substance plus some of the residual substance of the germinal vesicle. In addition there is nuclear chromatin from the first maturation division of the egg nucleus. The polocyte is entirely released from the egg, which immediately begins the second maturation division, the ectoplasmic defect remaining till this is complete. During this time the first polar body does not divide, as is the case in *Loligo,* but remains inactive adjacent to the outer membrane covering the egg. As in the case of the first polar body, the second contains none of the cortical ectoplasmic material but only endoplasmic cytoplasm and nucleus. The course as described here may be traced with ease by use of the cardioid dark field apparatus, as the egg is relatively small and there is an evident optical difference in the appearance of the cortical ectoplasmic and the deeper endoplasmic layers. Associated with this lack of the cortical layer is the fact that neither of the polocytes of *Chætopterus* divide as is the case of those produced by the egg of *Loligo.*

Discussion

As is well known, the relation between the formation of the polar bodies and the penetration of the spermatozoön varies in different groups of animals. In some forms the spermatozoön penetrates the egg before either of the polar bodies is formed. In other cases the first polar body is completed before penetration, but the second is dependent upon the activation of the egg at fertilization. In still another group the second polar body is also formed before penetration. It is evident that conditions controlling the development of the polar body must vary according to the conditions existing in different animals. In the case of the first group, the polocyte is a part of the egg at the time of the activation and therefore must initially be activated in the same way. In the second group the second polar body must be activated while the first is not. The first, in order to simulate the conditions existing in the polar body of the first group, must be penetrated by a sperm cell or be activated in some other fashion. In this group the second polar body might conceivably develop save for the fact that the cell organs introduced by the sperm are not present (see below). In the third group of eggs, neither of the polar bodies is a part of the egg at the time of sperm penetration and hence has fulfilled none of the conditions attendant on its activation. In order to show any development, therefore, it would have to be activated. In addition, as has already been mentioned by Conklin (1915) both of the polar bodies of the first group would be protected against further activation by sperm because of cortical modifications attendant upon the previous insemination of the egg. The same would be true of the second polar body in the second group, In order to explain the lack of development in these, it would be necessary to demonstrate either a consistent lack of some significant part of the nuclear or cytoplasmic portion of the entering spermatozoön or of the egg at the time of polocyte formation. In the same way, development of the second polocyte of the second group and both of the polocytes of the third group must be dependent on subsequent activation. The last statement is dependent on another condition which would seem in the light of the present observations to be of primary importance. The polocyte must contain all of the constituents of the egg essential both to its own activation, and to the activation of the spermatozoön.

Unfortunately, there is little information as to the actual constitution of the cytoplasmic portion of the polar body available in literature on the subject. These structures have been considered as aborted ova by Mark in his monograph on *Limax campestris* (1881). In that form the polar bodies do not develop. It would now be of great interest to know the actual constitution of the polocyte.

In isolated cases the polar bodies have been observed to develop to some extent at least. Lefevre (1907) cites the case of the polar body of *Thalassema mellita*, which is formed in response to the acid activation (artificial parthenogenesis) of the egg. Subsequent cleavage of the extruded cell gives rise to a ' morula,' which later dies. In this case also there is some confusion in that the actual composition of the polocytes which develop is not known. In no case did they develop beyond this early stage, however, though that may conceivably be due to quantitative deficiencies which do not immediately concern us here.

The polar nuclei such as are formed in some insects and crustacea do not come under the realm of our discussion, but, inasmuch as they sometimes cleave, we should mention them. The polar nuclei may fuse and in some cases they divide to form what appear to be accessory embryonic structures. This is the case in the hymenopter, *Litomastix* (Silvestri, 1908).

The most extensive development of the polocyte is to be found recorded in the paper of Francotte (1898) on the polyclads, in which he records the formation of the gastrula by fertilized first polar bodies of *Prosthecerœus vittatus*. The argument is slightly different from that in other cases, though it will concern us in a moment. We shall therefore consider it here. The egg of this form produces a polar body at the first maturation division, which may be as much as one-fourth as large as the remaining portion of the egg. Subsequently this may be fertilized, as is the egg itself, and then both of the units give rise to one polar body and continue in their development. Francotte described the development of these forms only as far as the gastrula. It may be assumed, however, that all of the conditions for cell division and development of any other than a quantitative nature are fulfilled. We shall return to a consideration of this case in connection with the fate of the so-called giant polar bodies of other forms.

In connection with the impregnation of the polar bodies after their extrusion, mention should be made of certain observations of Fol. This investigator has reported sperm penetration of polocytes in echinoderms. No mention is made of the reaction of the sperm or of the egg, so that it is not known as to whether the conditions of activation, as expressed morphologically, are complete or not. It is conceivable that in this case the very small size of the polar body would preclude its further development. It is also possible that the polar body of the echinoderm egg does not include all of the parts of the egg requisite to development.

It is in this last statement that we find a possible explanation of the difference between the behavior of the polar bodies in *Loligo* and *Chætopterus*. It can be demonstrated that in the formation of the polar bodies in *Chætopterus*, while the majority of the deutoplasm remains within the egg and the polocyte consists mainly of hyaloplasm, only the deeper endoplasmic portion of the cytoplasm is extruded, and the polocyte does not contain all of the zones characteristic of the egg which develops. It might be emphasized here that hyaloplasm and ectoplasm are not identical. In complete accord with this lack of ectoplasm, there is no second division of the primary polar body. In *Loligo*, on the other hand, the extruded polar body contains not only the endoplasmic region, but also some of the outer cortical part of the cytoplasm. In this case the polocytes develop for a little while. A striking difference between the two types of polar body is to be found in the presence or absence of the cortical ectoplasmic portion, which may be assumed, therefore, to play an important rôle in future events.

Certain observations made by Conklin (1915, 1917) on the behavior of artificial giant polocytes in *Crepidula* are very important in the consideration of the phenomenon. In this paper, Conklin changes his previous views on the subject and states that in such cases the lack of the sperm aster may be the important factor in the non-development of the large polar body. These were obtained by centrifuging the eggs during the formation of the polar body. In those cases in which the rotation of the egg was such that the spindle was in the line of the gravitational pull and at the outer end, huge polar bodies were formed which appeared to contain all of the egg substances and yet did not develop. Following certain previous conceptions, Conklin differentiates between those phenomena of fertilization leading to activation of the cytoplasm and to the development of the egg. The polocyte, having been activated with the rest of the egg by insemination, forms, but, lacking the aster which he considers as the part essential to further development, fails to divide. In view of the experiments of Lillie (1906) on *Chætopterus* eggs during maturation phases it would appear that there may be a question as to the *composition* of the polar body. If one is to examine Fig. 24 (p. 185) in Lillie's report, one sees that when the *Chætopterus* egg is centrifuged, certain substances of the egg are more or less free and that one of these is the endoplasmic material. As a result, the endoplasmic material takes its position according to the force of gravity. The ectoplasmic cortical material, on the other hand, is fixed, not being displaced by the centrifugal force. If, then, the force of gravity were applied in such a way that the endoplasmic material were all pushed against the region of the ectoplasmic defect or pole at which the spindle is attached, the result would be that it would bulge outside of the ectoplasmic portion at this point and that, when polar body formation was completed, a great quantity of this material would be separated from the egg and follow the polar body. This would not necessarily involve the inclusion of any of the ectoplasmic material in its formation. If this should prove to be the case, the non-development of the polar body of *Crepidula*, when present as the giant polar body, might be due to the same factors which seem at present to be responsible for the non-development of the polocyte of *Chætopterus*. In this connection it is of interest to note that the first polocyte of *Crepidula* may divide once by mitosis and subsequently several times by amitosis (Conklin, 1902, page 21, Fig. 41, etc.). This does not occur in the second polocyte. There may be a discrepancy in their constitution or in the quantitative relationships between the amounts of substances of cytoplasmic nature present. In this way the first polocyte may resemble that of *Loligo,* while the second may be like that of *Chætopterus*. In any event, the conditions present in *Chætopterus* do not obtain for *Loligo* and the results differ accordingly. The conception of the great importance of the cortical portion of the egg to the phenomena of early development is not new as it has been stressed in a number of places by several investigators, notably by Chambers (1921) and Just (1923). These authors show by their experiments that there is a real distinction morphologically and functionally between cortex and endoplasm.

In conclusion I would like to suggest that in *Loligo* and *Chætopterus* and possibly in other forms, the development of the polar body is dependent on the presence of cortical ectoplasmic material in that organ or at least on the ratio between the amount of the ectoplasmic substance and the remaining cytoplasmic and nuclear material.

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THE DISTRIBUTION OF PIGMENT AND OTHER MORPHOLOGICAL CONCOMITANTS OF THE METABOLIC GRADIENT IN OLIGOCHÆTS

GRACE EVELYN PICKFORD

Osborn Zoölogical Laboratory, Yale University

Introduction

The form of the antero-posterior metabolic gradient of the Oligochæts has now been well established by many workers and by almost as many different methods. Hyman (1916) investigated the gradient of susceptibility to KCN in many lower Oligochæts and distinguished two types, a primary gradient found only in the primitive Æolosomatidæ and young zoöids of the Naididæ in which the susceptibility decreased progressively from head to tail, and a widely distributed secondary type in which the susceptibility again rose at the posterior end. In *Lumbriculus varians* measurements of the oxygen intake of different regions of the body by the Winkler technique (Hyman and Galigher, 1921) showed a secondary metabolic gradient; the accurate manometric determinations of Shearer (1924) on the "earthworm" confirm the primary but throw no light on the secondary gradient, since the experiments were only made on head and tail portions. The early work of Morgan and Dimon (1904) on the potential gradient showed that in *Lumbricus terrestris* and *Allolobophora fœtida* the head and tail were electronegative to the middle region, while Moore and Kellogg (1914) found that in an electric field *Lumbricus* oriented itself in the form of a U with head and tail towards the cathode. Hyman and Bellamy (1922) confirmed these results and correlated them with the metabolic gradient. Hatai (1924) showed that in two Japanese species of *Pheretima* (incorrectly named *Perichæta*) the amount of heat required to produce initial heat rigor in the muscles of the body wall was greatest at the anterior and posterior ends and least in the middle region of the body. He correlated these results with the percentage water content of the body wall, which is inversely proportional to the temperature required to produce initial heat rigor. Watanabe (1928) found that in *P. communissima* the potential gradient is on the average of the secondary type, although dorsally it is perhaps of the primary type.

Recently Perkins (1929) has published a short note in which he claims that in earthworms the gradient of extractable reduced sulphydryl reaches a maximum in the mid anterior region of the body. Perkins summarizes his results as follows, "In earthworms I find that the gradient of growth corresponds with the gradients of total iodine equivalence, extractable sulphydryl, and total sulphur (gravimetric) and not with the gradient of total metabolism observed by the oxygen uptake; the last, therefore, includes other oxidation systems which it is legitimate to suppose result in katabolism rather than the anabolism of growth. It is interesting to find that gradients in the earthworm have a summit at about that point whence a divided worm grows forwards or backwards according to the aspect of the cut surface."[1]

As regards the dorso-ventral gradient very little work has been done, although Hatai (1924) states that the temperature necessary to produce initial heat rigor is greater for dorsal than for ventral and intermediate for lateral portions of the body wall.

Little attention has been paid to the morphological concomitants of the metabolic gradient. Hess (1924) showed that the sensitivity of *Lumbricus terrestris* to light is greatest at the anterior end and least in the mid region of the body and that except on the first five and last two segments it is confined to the dorso-lateral regions; he also noticed that the distribution of pigment corresponds rather closely to the light sensitivity. In a later paper Hess (1925) showed that the distribution of the photo-receptor organs coincides with the distribution of the photosensitive regions, thus putting the gradient of sensitivity to light on a morphological basis. Nomura (1926) has extended the work of Hess, showing that in the ventral nerve cord of *Allolobophora fœtida* Sav. there is an axially graded distribution of photic response; negative orientation, which also characterizes the brain, increasing posteriorly and positive orientation anteriorly, while the supposed neurones causing backward crawling are apparently restricted to the anterior end opposing the brain, which controls forward crawling.

Distribution of Pigment

Many species of earthworm are pallid and others may be colored green, blackish, or yellow by as yet uninvestigated pigments, but by far the most commonly occurring coloration is due to a reddish or purplish-brown pigment which has been shown in some species (see Kobayashi, 1928) to be a porphyrin allied to some derived from chlorophyl. This reddish pigment is characteristically distributed on the dorsal side and is most intense at the anterior end. A typical case can be found in the well-known species *Lumbricus terrestris* Linn. Indi-

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[1] References to text figure omitted.

viduals of this species will be found to vary somewhat in the intensity and exact extent of pigmentation, but the following description taken from a specimen recently caught near this laboratory will serve as an example: "Intensely pigmented dorsally at the anterior end, the pigmentation extending laterally to about *cd* (the line of the lateral setæ), the first three segments also slightly pigmented ventrally; posterior to the clitellum the lateral extent of the dorsal pigmentation becomes reduced until only a mid-dorsal line is left which persists throughout the posterior half of the body; at the extreme posterior end there is again an increase in intensity and extent of pigmentation (except on the terminal segment which is small and pale) which extends laterally to below the setal line *cd* on the seventh to the second last segments and even faintly on the ventral side of the second, third, and fourth segments from the end." In this case the distribution of pigment follows the secondary type of gradient, and it may be said in general that whenever a species of earthworm exhibits this red-brown pigmentation (presumed, but of course not proved in most genera to be due to a porphyrin allied to that of *Lumbricus* and *Allolobophora* (*Eisenia*) *fœtida* Sav., it will be distributed according to the primary if not the secondary type of gradient. Hatai (1924) noticed that in "*Perichæta*" *megascolidioides* Goto et Hatai the dorsal side was more pigmented than the ventral, though curiously enough he did not correlate this with the dorso-ventral gradient. My own investigations have so far been confined to a systematic examination of South African species of the genera *Chilota* and *Acanthodrilus*. In these genera every gradation from total pallor to intense pigmentation can be found; some of the most interesting cases are those species, or varieties of otherwise pallid or pigmented species, in which pigmentation is only found on the first or last few segments. For example, the Cape Flats species *Acanthodrilus arundinis* Bedd. is pigmented dorsally on the first and last four or five segments but more intensely on the latter, while in many undescribed species of *Chilota* only a few of the anterior segments are pigmented. When the dorsal pigmentation is intense and occurs along the whole length of the body it is usual to find that the first five to ten segments are deeply pigmented ventrally, while in many cases pigment is deposited on the thickened septæ and generally on the inner side of the body wall at the anterior end. A more complete discussion will be given in my forthcoming paper on the South African Acanthodrilinæ.

The distribution of pigment in Oligochæts may be compared with that described by Faris (1924) for *Amblystoma* embryos. In this case the pigment is apparently a melanin and is deposited in regions of tissue differentiation as opposed to regions of proliferation. If the intensity of pigmentation in Oligochæts is really a function of the metabolic rate, it seems possible that highly pigmented species would have a higher oxygen intake than pallid ones. It is hoped to investigate this point shortly on a large number of species. If this view is correct, and it is supported by the fact that pallid species are more sluggish in their movements and less irritable to handling than pigmented ones (compare *Allolobophora* (*Eisenia*) *rosea* Sav. with species of *Lumbricus*), it would seem unlikely that the porphyrin is merely derived from the food of the worm, as has been suggested, and more probable that it is a breakdown product of the worm's own hæmoglobin.

MULTIPLICATION AND REDUCTION OF SETÆ

As regards the more specifically morphological concomitants of the axial gradient, certain stages in the reduction and multiplication of setal numbers are significant. In the primitive lumbricine condition there are two pairs of setæ per segment except on the first, which never has setæ; a reduction in numbers sometimes takes place as in species of the *Microchætus benhami* group where setæ are absent on the first six or seven segments of the adult (frequently only the lateral pair are absent on segment 6). This trend to reduction finds an extreme case in *Tritogenia crassa* Mchlsn., in which only the ventral setæ of the clitellar region persist.

In the Enchytræidæ parallel cases can be found; in the genus *Distichopus* only ventral setæ are present. In the genus *Michaelsena* transitional species occur from *M. mangeri* Mchlsn., in which dorsal and ventral setæ are present throughout and *M. principissæ* Mchlsn., in which the ventral setæ commence on segment 3 and the dorsal on segment 14, to *M. normani* Mchlsn., which has ventral setæ from segment 3 onwards but dorsal setæ only on segments 4–6, and *M. subtilis* Ude., in which dorsal setæ are absent and ventral setæ occur only on segments 4–6. In the genus *Achæta* setæ are totally absent. These cases may be compared with the phenomenon of cephalization in the Naididæ (Stephenson, 1912 and 1923), in which certain anterior segments are devoid of dorsal (*i.e.* lateral) setæ. Hyman (1916) found a very peculiar gradient in the Naid *Chætogaster diaphanus*, in which the susceptibility was least at the head end. In this genus dorsal setæ are totally absent and ventral setæ though present on segment 2 are absent on segments 3, 4, and 5.

The tendency to setal multiplication is a very widely distributed phenomenon, and the perichætine condition has apparently arisen independently many times in various families of the terrestrial or Neo-Oligochæts (see Stephenson 1921, 1923 for a discussion of this and other trends in the evolution of the Indian Oligochæta). The multiplication of setæ varies from a condition in which six or eight pairs occur instead of four per segment to the purely perichætine condition in which each segment has a complete ring, but the most interesting cases are those in which a transitional condition exists. In *Megascolex willeyi* Mchlsn. there are eight setæ per segment at the anterior end and twelve in the middle and posterior regions; in *M. vilpattiensis* Mchlsn. there are eight setæ in four pairs on segments 2 and 3, eight or nine on segment 4, *circa* 11 on segment 13, *circa* 24 on segment 26, and *circa* 26 at the posterior end. In general in transitional species the smaller number and 1 or more primitive paired condition persist at the anterior end. Sufficient data are unfortunately not available as to the extreme posterior end, so that it is not possible to state whether the smaller number also persists there in these intermediate forms. Hatai (1924) has investigated the setal numbers in the purely perichætine species "*Perichæta*" (*Pheretima*) *megascolidioides* Goto et Hatai. He finds that the number of segments is extremely constant and bears no relation to the size of the worm and that the total number of setæ per worm does not vary very greatly. The number of setæ per segment increases from segment 2–25, remains about constant up to segment 100 and then decreases again, thus exhibiting a curve comparable with the secondary type of oligochæt gradient. From a survey of the available data it would thus seem as if setal multiplication were correlated with a lower and setal reduction with a higher metabolic rate. The case of *Acanthobdella peledina* Grube, an aberrant parasitic form regarded until recently as a leech, must not be overlooked, although the evidence (c.f. *Chætogaster*) cannot be interpreted until the form of the metabolic gradient has been investigated. In this species setæ are present only ventrally on the first five segments.

Multiplication and Reduction of Nephridia

The trend to setal multiplication is paralleled and usually accompanied by the multiplication of the nephridia, primitively one pair per segment. Unfortunately the whole subject of nephridial multiplication stands in need of a thorough revision since the publication of Bahl's admirable series of studies on *Pheretima* (1919 and 1922), *Lampito* (1924) and *Woodwardia* (1926). The brief descriptions of systematists who classified their species as "micronephridial," "meganephridial," and "mixed mega-and-micronephridial" are now shown to be totally inadequate. Nevertheless, what little can be judged from the existing knowledge yields points of considerable interest. In the first place, loss or reduction of nephridia when it occurs seems to take place at the anterior end, *e.g.* in *Pontodrilus, Sparganophilus* and *Diporochæta pellucida* Bourne (*re* last species see Stephenson, 1925). Bahl considers that the first step in nephridial multiplication was the separation of the nephrostome, which then either disappeared or formed with accompanying nephridial cells a separate septal meganephridium opening into the gut, while the main mass of the nephridium broke up to form funnel-less integumentary nephridia. In *Pheretima* the septal nephridia have also undergone multiplication to the micronephridial condition. If this view be provisionally accepted, the two trends, separation of the nephrostome and multiplication, may be considered independently. As regards the former, numerous cases can be found in the literature in which "meganephridia" occur only in the middle and posterior regions of the body. In "*Lampito*" (*Megascolex*) *trilobata* Steph. and "*L.*" *mauritii* Kinb., Bahl found that the septal meganephridia commenced in segment 19, while in *Woodwardia bahli* Steph. they commence at 24/25. Benham (1905) describes two species of *Spenceriella,*—"*Diporochæta*" *gigantea* and "*D.*" *shakespeari*, which are "micronephric" but retain large paired nephrostomes in each segment. Unfortunately he does not say how far forward these occurred. In *Comarodrilus gravelyi* Steph. "micronephridia" occur in the anterior part of the body as far back as segment 12; behind this "meganephridia" only. In the development of *Octochætus multiporus,* Beddard found (1892) that the nephrostomes degenerate after their separation from the nephridial mass, but that they may persist in the posterior segments. These cases appear to be merely examples of a very general phenomenon, viz., the tendency for the nephrostomes to disappear anteriorly. An interesting case is that of *Howascolex corethrurus* Mchlsn., a species which is transitional both for perichætine and micronephridial conditions. The setæ are lumbricine in the anterior and middle regions and perichætine posteriorly, while "meganephridia" displace the "micronephridia" posteriorly.

The case of nephridial multiplication *sensu stricto* requires a statistical investigation, but observations such as those of Bahl on "*Lampito*" and *Pheretima* spp. and of Stephenson on *Hoplochætella kinneari* Steph. indicate that a great multiplication in numbers of micronephridia in the clitellar region may be a general phenomenon.

While there is thus considerable evidence that nephridial and nephrostomal reduction follows the primary metabolic gradient, occurring first at the anterior end, the case of nephridial multiplication is not at all clear cut and the issue is frequently confused by the occurrence of pharyngeal nephridia (tufts of funnel-less nephridia opening into the pharynx) in the most anterior segments. The clitellar region, which is sometimes the region of greatest multiplication (*vide supra*), is not known to be the region of lowest metabolism, since the physiological gradient has not been investigated for the species concerned, but evidence from other species suggests that the clitellar region is too far forward to coincide with the region of lowest metabolism. If Perkins' (1929) speculations as to the anabolic gradient are well founded, it is possible that certain morphological features such as nephridial multiplication in the clitellar region might be interpreted more readily by a correlation with this rather than with the total metabolic gradient. Examples have been cited above in which "micronephridia" are replaced by or co-exist with "meganephridia" in the posterior part of the body. Sometimes, *e.g.*, in *Notoscolex palniensis* Steph., these "meganephridia" are definitely stated to be enlarged "micronephridia" without funnels (Stephenson, 1924). Cases of nephridial multiplication without separation of the nephrostome are extremely rare. Bahl (1926) has described the case of "*Lampito*" *dubius* Steph., and apparently a similar phenomenon occurs in the genus *Tritogenia*, which has two pairs of nephridia per segment. In "*Lampito*" *dubius* there are five pairs of septal exonephridia per segment except anteriorly, where there may be only three pairs. On the whole there is a suggestion that nephridial multiplication is less pronounced in the regions of highest metabolism.

Homœosis

Finally, I should like to draw the attention of zoölogists who have not made a study of oligochæt systematics to the very general occurrence of homœosis, not merely as occasional variations (Bateson, 1894) but as normal subspecific, specific, generic and family characters, the segmental shifting forwards and backwards of various organs, *e.g.* the clitellum, genital openings, and accessory glands, gizzard, etc. being of prime taxonomic importance. An excellent example may be taken from the genus *Acanthodrilus*, which normally possesses paired male pores on segment 18 and two pairs of prostatic pores on segments 17 and 19; there may, however, be a backward shifting (Michaelsen, 1913) as in *Ac. coneensis* Mchlsn. and *Ac. natalicius* Mchlsn. with the male pores on segment 19 and prostatic pores on 18 and 20 or *Ac. rouxi* Mchlsn. with the male pores on segment 20 and the prostatic pores on 19 and 21. A similar phenomenon occurs in undescribed South African species of *Chilota*.

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SUMMARY

In Oligochæta the distribution of the photoreceptor organs and of porphyrin pigmentation as well as the tendencies to reduction and multiplication in numbers of setæ and of nephridia per segment appear as morphological concomitants of the metabolic gradient.

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DISTRIBUTION OF SETÆ IN THE EARTHWORM, *PHERETIMA BENGUETENSIS* BEDDARD [1]

P. B. SIVICKIS

(From the Zoölogical Laboratories, University of the Philippines, Manila and Lietuvos Universitetas, Kaunas, Lithuania.)

The oligochæt genus, *Pheretima,* which occurs abundantly in the Philippines and other oriental countries, is characterized by the presence of a large number of setæ on each segment except the most anterior. Taxonomists have regarded the distribution and number of setæ as specific characteristics, but apparently have observed that the number varies on different segments, since they usually specify the segment for which the number of setæ is given (Michaelsen, 1900; Stephenson, 1923). No data have been found, however, concerning variation in number of setæ on a particular segment. Counts of setæ made by the writer show a considerable range of variation, both in the number of setæ on corresponding segments of different individuals and on different segments of the same individual. Moreover, the numbers of setæ on different segments of the same individual vary along the axis in a way which suggests a relation to the longitudinal physiological gradients. Data are given below concerning these variations.

Material and Methods

Pheretima benguetensis Beddard, the species on which the counts were made, is common in the Philippines. During the greater part of the rainy season the worms are found in large numbers near or on the surface of the ground. By the end of the rainy reason they become heavily parasitized by gregarines and later disappear almost completely, but whether the disappearance is due to death or to movement away from the surface of the ground is not known.

Counts of setæ were made on one hundred animals. Fifty of these were collected on the campus of the University of the Philippines and fifty from the town of Pasig near Manila. The latter were somewhat larger than the former, but their general specific characteristics indicated that both lots belonged to the same species.

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[1] The data presented in this paper were obtained while the writer was a member of the Department of Zoölogy of the University of the Philippines. Acknowledgments are due to Miss Paz Lorenzo, Mr. D. Quajunco and Mr. G. T. Lantin for assistance. My thanks are due to Prof. C. M. Child for critical review of this paper.

The counts were made on animals preserved in formalin. For counting they were opened along the mid-dorsal line, the internal organs were removed, and the body wall was cut into pieces of a size convenient for microscopic examination between two slides. Counts of such pieces were either made at once or the two slides with the piece between them were tied together and placed in a hot one per cent solution of KOH for five hours or more, until they became transparent, but were removed before maceration had proceeded so far that the setæ were freed from the tissue. A section along the dorsal midline is more satisfactory for such preparations than a section elsewhere because the dorsal wall is thicker than in other regions, and since the KOH attacks the edges of the preparation first, the thicker dorsal wall is not destroyed before the other parts have become sufficiently transparent. After maceration the pieces were mounted in glycerol and all the setæ on the segments selected were counted under a low power of the compound microscope with the aid of a mechanical stage. Particular care was taken to make certain that all setæ on each segment selected were included in the counts. In the region of the clitellum counts are less readily made than elsewhere because the thickening of the body wall in this region makes it difficult to see the setæ.

Since there are no setæ on the first segment, counts were begun with the second, and further counts were made on the fifth, tenth, fifteenth, etc., that is, on every fifth segment up to the sixtieth. In order to minimize possible errors which, however, proved to be less than was feared, in counts on the fifteenth segment, a segment of the clitellum, counts were made on the segment next anterior (13) and the segment next posterior (17) to the clitellum. Counts from the posterior direction began with the last posterior segment and were made on every fifth segment until the sixty-fifth segment from the posterior end was reached. This procedure leaves a short middle region uncounted in some animals with a large number of segments, but since the mean number of segments can readily be extrapolated in this region, the results are not seriously affected.

The method of making counts in two directions from each end of the body is regarded as preferable to that of making counts from anterior to posterior end, because by the latter method the most posterior segment counted is rarely the last segment of the body and represents different levels in different cases.

Distribution and Size of Setæ

Each segment except the most anterior possesses a large number of setæ more or less uniformly distributed about the circumference, but with occasional gaps and occasional duplications. The setæ are less than a millimeter in length, and taper slightly from the base to a blunt tip.

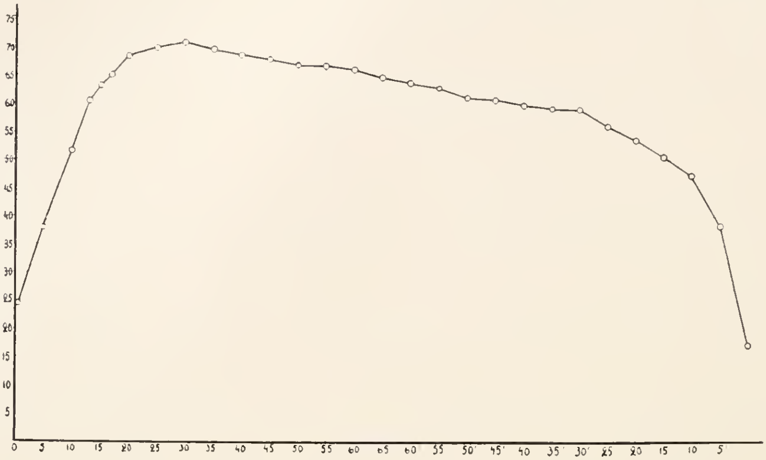

FIG. 1. Graph from the data of Table I showing the variation in numbers of setæ along the main axis of the body in *Pheretima benguetensis*. Ordinates represent the mean numbers of setæ (M) on particular segments; abscissæ represent segment numbers. The anterior end is at the left.

Setæ from three regions of the body have been isolated by boiling in KOH pieces of the body wall from the selected regions and have been measured with an ocular micrometer. The data of such measurements are as follows:

On segments	2–5,	length	0.6 mm.;	diameter	0.08 mm.
" "	36–40,	"	0.3 mm.;	"	0.05 mm.
Last ten segments,		"	0.5 mm.;	"	0.03 mm.

These measurements indicate the variation in size of the setæ. The longest setæ are found on the anterior and posterior segments, the shortest in the middle regions. From the anterior end the setæ very gradually decrease in size to the clitellum. For some distance posterior to the clitellum the setæ are only about half the length and little more than half the diameter of those on the anterior segments. Posterior to the middle of the body they begin to increase in size and for the most posterior segments they are almost as long, though less in diameter than those at the anterior end. In general the length of the setæ varies inversely as their number.

TABLE I

Numbers of Setae on Selected Segments of First and Last Ten Individuals of Pheretima Benguetensis out of One Hundred Counted. The last two horizontal lines give the mean values (M) and standard deviations (σ) for corresponding segments of one hundred animals.

Worm Number	Segment Numbers																													Number of Segments
	2	5	10	13	15	17	20	25	30	35	40	45	50	55	60	65′	60′	55′	50′	45′	40′	35′	30′	25′	20′	15′	10′	5′	1′	
1	22	33	52	48	53	54	65	67	69	72	70	74	65	60		55	52	53	51	50	50	53	41	45	40	41	38	30	22	128
2	24	39	49	55	60	65	67	69	71	74	69	66	69	65		54	54	57	55	51	53	53	47	45	48	42	43	35	22	127
3	21	33	46	50	50	58	66	64	65	60	58	58	62	58	53	54	54	56	50	54	47	52	47	48	49	48	40	36	11	133
4	30	43	48	56	60	60	74	70	74	76	76	71	72	69	72	69	67	65	66	70	68	67	65	61	54	53	51	43	17	130
5	34	43	55	65	69	70	75	75	76	75	74	72	70	69	70	74	76	75	69	69	71	70	64	61	64	60	55	46	12	131
6	22	33	45	58	56	58	61	64	62	59	55	58	54	57	61	58	59	55	53	52	55	49	49	46	47	42	44	34	7	134
7	23	33	50	56	58	59	62	66	67	60	55	54	60				59	61	58	58	59	60	58	58	57	50	46	42	15	115
8	20	42	50	68	70	75	75	77	76	75	71	74	73	71	73	68	68	65	64	66	62	60	63	62	60	54	49	44	10	134
9	25	37	46	59	60	61	62	65	66	60	64	68	65	66	60	60	57	58	56	58	57	57	56	51	50	50	47	38	11	134
10	20	40	42	52	54	58	68	66	65	64	66	65	67	64	63	58	60	58	56	55	50	56	59	58	58	52	50	32	12	132
91	27	40	58	67	68	63	81	76	78	77	76	80	76	73	74	72	71	68	65	72	67	66	63	59	57	53	51	40	28	134
92	24	39	48	60	58	61	60	61	63	62	65	72	74	71	69	72	67	69	71	68	63	65	60	57	52	47	40	31	17	136
93	17	35	49	62	66	72	76	81	74	69	68	73	71	69	71	68	70	67	70	69	65	61	61	59	56	48	45	42	22	132
94	21	39	53	58	61	67	65	72	75	72	72	68	65	68	67	67	69	71	70	63	60	63	61	58	54	51	35	32	7	140
95	18	42	46	60	67	69	73	75	67	74	71	66	67	70	69	68	68	65	67	68	62	61	65	59	60	52	47	29	14	128
96	29	38	65	66	70	73	82	79	78	74	76	76	78	80	76	74	69	66	65	63	61	60	59	62	56	47	33	27	12	140
97	24	37	60	62	65	66	72	73	75	72	70	71	74	71	66	65	68	64	66	61	59	57	58	53	56	54	46	41	10	133
98	22	35	54	65	67	77	81	74	76	77	80	71	68	71	71	68	70	68	66	60	63	65	62	55	57	49	37	32	20	138
99	24	34	48	55	57	62	71	70	68	68	66	65	63	61	52	59	62	60	63	61	58	55	52	51	49	46	45	35	9	134
100	27	44	59	63	67	74	77	76	79	75	73	76	72	69	68		67	65	63	58	54	52	47	48	43	46	39	35	16	122
M	24.5	38.0	51.8	60.8	63.4	65.3	68.5	69.9	70.9	69.5	68.7	67.8	66.8	66.8	65.9	64.8	63.7	62.9	61.0	60.8	59.7	59.0	59.0	55.7	53.6	50.8	47.3	38.4	17.4	131.0
σ	3.59	3.02	5.07	5.29	4.62	5.39	6.29	5.68	6.00	5.69	5.56	5.86	5.64	5.17	4.81	5.24	5.38	5.23	5.24	5.49	5.60	4.64	5.18	4.85	5.07	4.98	5.39	6.60	9.68	5.02

COUNTS OF SETÆ

The numerical data for the first ten and the last ten animals of the hundred counted are recorded in full in Table I as a sample indicating how the counts run. Animals 1–10 of the table are from those collected on the University campus, animals 91–100 from those collected at Pasig. The first vertical column gives the number of the individual in the series, the following column the number of setæ counted on corresponding segments. The first vertical section of the table gives counts from the anterior end to the sixtieth segment, the second section, counts from the posterior end to the sixty-fifth segment from that end. The last column gives the number of segments in each animal. In the last two horizontal lines of the table are given the mean values (M) and the standard deviation (σ) as calculated by the standard formulæ for corresponding segments of all animals counted, that is, each value of M and σ given is the value for one hundred corresponding segments. The variations of M at the different body levels are plotted in the graph (Fig. 1).

Examination of the data recorded in the table shows that in spite of a considerable variation in the number of setæ per segment of any individual, the general course of the variations in different regions is well expressed by the means. The number of setæ is relatively small on the anterior segments, but increases rapidly to the twentieth segment, beyond this more slowly to the thirtieth segment, where the maximum number of setæ per segment is attained. Posterior to this segment the number of setæ decreases gradually to the posterior end of the body.

DISCUSSION

The very definite course of variation in number of setæ along the body of *Pheretima* suggests that it must be correlated with regional physiological differences of some sort, and since it is gradual and in opposite directions in anterior and posterior regions, the possibility that it may be in some way correlated with the longitudinal physiological gradient in the body is also suggested. Nothing is known concerning the gradients in *Pheretima,* but in most other oligochæts examined a double gradient has been found. Hyman (1916) has found in most of the microdrilous oligochæts a decrease in susceptibility from the anterior end posteriorly to a certain level and an increase from this level to the posterior end. Hyman and Galigher (1921) found a similar double gradient in oxygen consumption in *Lumbriculus* and *Nereis.* Perkins (1929), investigating oxygen consumption, total iodine equivalence, amount of glutathione and total sulphur content in different regions of the body of an earthworm (unnamed), also finds differentials which vary in two directions. If such a double gradient exists in *Pheretima,* as is probable, the smaller numbers of setæ occur at the higher, and the larger numbers at the lower levels of the respiratory gradient. We know nothing at present concerning the nature of the relations between gradients and setæ, but it may be provisionally suggested that a developing seta sac in the regions of more intense metabolism inhibits the development of other seta sacs over a greater distance than in regions of lower metabolism, consequently at the higher gradient levels fewer setæ develop on the circumference of the segment than at lower levels. Such an inhibiting action of a developing part or organ on other similar organs within a certain distance from it is very generally recognized by both botanists and zoölogists, and in various cases the range of this effect appears to be very definitely associated with the intensity of metabolism in the part concerned. Whether this suggestion of a possible relation between the numbers of setæ on different regions of the body is correct must remain for further investigation to determine.

In addition to the regional variations in numbers of setæ, individual variations in number on corresponding segments appear in the table. The standard deviation σ is lowest in the anterior region of the body. This is particularly evident anterior to the tenth segment. The highest value of σ appears in the posterior region, particularly in the ten posterior segments. Between these extremes σ fluctuates between 4.62 and 6.00. The relatively low σ of the anterior region suggests physiological stability in this region, and this is in accord with the fact that it develops first and represents a dominant or relatively dominant region. It is much less affected by parts posterior to it than they are by it.

With respect to the practise of taxonomists of considering the number of setæ on a particular segment as a specific character, it may be noted that the data presented in the table show a very considerable individual variation in these numbers and a high value of σ. Apparently counts on many individuals would be necessary to make these numbers reliable for species determination. Smaller numbers may, however, be considered as possessing a certain diagnostic value when considered together with other characters.

Some observations on *Pheretima posthuma* (*P. incerta* Beddard) indicate that with certain limitations similar relations exist in that species.

SUMMARY

The numbers of setæ on particular segments of *Pheretima benguetensis* vary in definite directions in different regions of the body. The number is lowest on the most anterior segments, increases posteriorly to a maximum at a level just posterior to the reproductive organs, and then decreases gradually to the posterior end. A relation between this course of variation and the physiological gradients is suggested. The standard deviations for corresponding segments indicate that the number of setæ on a particular segment should be used for determination of species only in connection with other characters.

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STUDIES ON THE PHYSIOLOGY OF THE EUGLENOID FLAGELLATES

II. The Autocatalytic Equation and the Question of An Autocatalyst in Growth of *Euglena*

THEODORE L. JAHN

Biological Laboratory, University College, New York University

The theory of a catalyst of growth, as proposed by Robertson, has been the stimulus for a number of investigations to determine the presence or absence of an autocatalyst in protozoan cultures. The earlier investigations have been reviewed previously (Jahn, 1929), and it was shown experimentally at this time that the growth rate of *Euglena* in mass cultures of high concentrations of organisms was not higher than in cultures of relatively low concentrations, but that in most cases the reverse was true.

It is the purpose of the present paper to reanalyze the experimental data previously obtained (Jahn, 1929) from the point of view of relative rate of division at various times during the period of observation. It will be shown, first, that the division rate, as calculated from the autocatalytic formula used by Robertson in his work on ciliates, is a progressively decreasing quantity, and hence that this autocatalytic equation can not be interpreted to involve an autocatalyst which effects an increase in division rate; and second, that, on the basis of experimental evidence, the autocatalytic equation may fit the growth curve of *Euglena* cultures. On the basis of the experimental evidence, it is believed that Robertson's theory of an autocatalyst of growth is unnecessary to an interpretation of the experimental data obtained in the case of *Euglena*.

The writer is deeply indebted to Professor R. P. Hall for suggestions offered during the preparation of this paper.

The Division Rate as Derived from the Autocatalytic Equation

The equation for an autocatalytic chemical reaction has been applied by Robertson (1923) to the rate of growth of ciliates in isolation cultures. The differential form of the equation is

$$\frac{dx}{dt} = Kx(A - x), \qquad \text{(Equation 1)}$$

where x is the number of organisms, A the maximum number attainable in a given amount of medium in question, and t is time. When integrated this becomes

$$\log \frac{x}{A - x} = AK(t - t_1), \qquad \text{(Equation 2)}$$

where t_1 is the time when $x = A/2$.

The differential equation states that the rate of increase in number of organisms at any time is proportional to the number present at that time and to the difference between that number and the maximum number attainable. Or one may let A equal the original (and also total) food supply. If this is measured in units, such that one organism utilizes on the average exactly one unit of food between divisions, then at any time the amount of food consumed will be equal to the number of divisions that have taken place. After the first few divisions the number of divisions which have occurred is approximately equal to the number of organisms present. Therefore A-x may be regarded as the available food supply, and in this sense the equation means that the *rate of increase in number* is proportional to the number present and to the amount of free food material. In either case dx/dt is the *rate of increase of the total number* of organisms as related to time. This, however, is interpreted incorrectly by Robertson as the *rate of division* of the organisms. The actual *rate of division,* that is, the average frequency of division (or fission) per unit time, is not dx/dt but $\frac{dx/dt}{x}$, or the rate of increase of the total number of organisms divided by the number (x) present at any given time (t). This we may represent by D, and then we may restate the division rate as

$$D = \frac{dx/dt}{x} = \frac{Kx(A - x)}{x} = K(A - x). \qquad \text{(Equation 3)}$$

The division rate therefore varies directly as A-x. Since A is constant, and x is continually increasing, and A-x therefore decreasing, it can readily be seen that the division rate as derived from the autocatalytic equation is a decreasing linear function of x. If the division rate is plotted against time, the result will be a sigmoid curve with a negative slope, practically the same as the original integral curve except that the abscissa will be shifted and the ordinate sign reversed (Fig. 1). Since the division rate is continually decreasing, the intervals between divisions will become progressively longer as the culture is continued.

More recently Robertson (1928) has proposed a new equation for the growth of Metazoa. The differential form of this equation is

$$\frac{dx}{dt} = \frac{k\rho}{1+\rho}(x+b)(A-x), \qquad \text{(Equation 4)}$$

where ρ is the constant proportion between nuclear and cytoplasmic increment and b/ρ is the excess of nuclear material which is present at the initiation of development, that is at the moment of fertilization, b being the same quantity translated into its cytoplasmic equivalent through multiplication by the proportionality factor ρ. Whatever meaning ρ could assume in a protozoan culture is difficult to state, but the division rate as calculated from this new equation is also a decreasing quantity as in the previous set of equations.

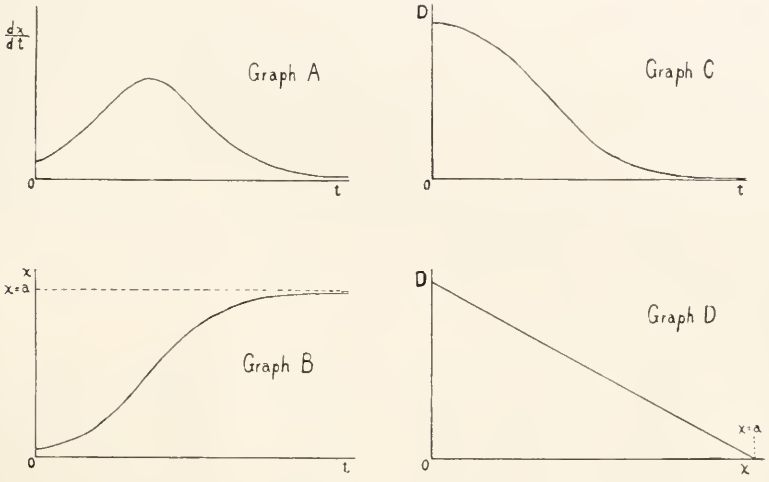

FIG. 1. Type curves computed from the autocatalytic equation.

A. Differential curve showing dx/dt, the rate of increase in numbers, plotted against time (t).
B. Integral curve showing x, the number of organisms, plotted against t; x approaches the value $x = a$ as an asymptote.
C. Division rate (D) plotted against time. D approaches zero as an asymptote.
D. Division rate plotted against numbers of organisms (x). D is a decreasing linear function of x, becoming zero when x is equal to a. The scale of t is the same in graphs *A*, *B*, and *C*.

$$D = \frac{dx/dt}{x} = \frac{k\rho A b}{(1+\rho)x} + \frac{k\rho}{1+\rho}(A-b-x). \qquad \text{(Equation 5)}$$

The division rate as calculated from the new equation is equal to the sum of two quantities, one of which varies as the reciprocal of x and the other as a decreasing linear function of x. The sum of these two quantities is, of course, a decreasing function. Therefore, the division rate, whether calculated from the new or from the old equation, is a decreasing function.

The above modification (equation 3) of the autocatalytic equation has been expressed by Brody (1927), who states, "It signifies that the relative rate of growth is directly proportional to the growth impulse," $(a\text{-}x)$. Since Brody was considering the application of the formula to Metazoa, he did not express the idea that the modification might be used to represent division rate of cells, or that under such conditions it would indicate a decrease in division rate.

Snell (1929) points out that since the volume of a growing organism changes, equations derived from the law of mass action can not be applied to growth without considerable modification. The value of $\frac{dx/dt}{x}$ calculated from the modification he proposes is also a decreasing function as in the preceding equations.

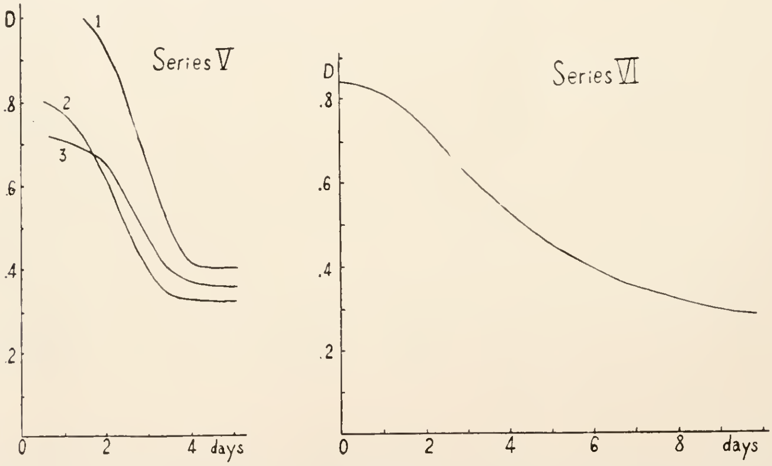

FIG. 2. Graphs showing the division rate (D) plotted against time for the three cultures of Series V and a composite curve for the three cultures of Series VI. Values of D were computed from the equation

$$D = \frac{dx/dt}{x}$$

The rate of increase of the total, dx/dt was determined by the graphical differentiation of the experimental growth curves. These values were then divided by the corresponding values of x to give the values of D for the times (t) under consideration. It is to be noted that this is a descending sigmoid curve such as is to be expected if the autocatalytic equation is applicable to the case. (See also graph C, Fig. 1.)

THE DIVISION RATE OF *Euglena*

The growth curves of *Euglena* from four series (I, III, V, and VI) previously described by Jahn (1929) have been differentiated graphically to give values of dx/dt for various values of t. If the values of dx/dt are divided by corresponding values of x, one may arrive at values of $\frac{dx/dt}{x}$, or D, for the values of t considered. If D is plotted against t, the result is a descending sigmoid curve. The division rate curves for Series V and VI are shown in Fig. 2; the curves for the other series are similar in form.

In Table I are shown the values of the division rate as computed from analysable data.

TABLE I

Culture number	Value of $\frac{dx/dt}{x}$ for Mid-day				
	Day				
	1	2	3	4	5
I, 1	.63	.36	.33	.31	—
III, 1	.80	.59	.44	—	—
V, 1	—	1.00	.80	.50	.40
V, 2	.80	.73	.50	.43	.34
V, 3	.72	.69	.55	.40	.37
VI, 1, 2, and 3 (averaged)	.83	.79	.66	.55	.50

DISCUSSION

The results of the above analyses demonstrate a decreasing sigmoid division-rate curve for cultures of *Euglena*. This indicates that the growth rate of *Euglena* closely simulates the reaction rate expressed by the autocatalytic curve, and that the periods between cell divisions in a single line of cells become progressively longer as the culture is continued.

The writer's observations on *Euglena* thus differ from those of Robertson (summary, 1924), who maintains that his experiments also show the growth of Infusoria (*Enchelys*) to be autocatalytic, since in isolation cultures the division rate is low at first but becomes progressively higher with each successive division. The autocatalytic formula, as stated, can be adopted only on the assumption that the food supply is limited from the beginning and is therefore continuously being decreased by the growth of the organisms. In Robertson's experiments the *available* food supply was not decreasing during the period of observation but was *increasing* due to bacterial growth, and Robertson's cultures also show an *increase* in division rate and not a decrease as required for the application of the *autocatalytic equation*. Hence, it is obvious that the autocatalytic growth curve cannot be applied to such experiments with ciliates.

The experiments of the writer with *Euglena* were conducted under more readily controlled conditions than were previous experiments with ciliates. Since bacteria are not a source of food for *Euglena*, it is safe to assume that the few bacteria present did not accelerate appreciably the division rate of the organisms. Therefore, the food of the flagellates was limited to the inorganic salts initially present in the medium and the carbon dioxide dissolved in the water. Since the primary physical factors (light and temperature) affecting growth were constant, and the chemical substances (carbon dioxide and inorganic salts) entering the reaction were continuously decreasing as the reaction progressed, the experiment may be considered as more nearly resembling a closed system—such as that to which the autocatalytic equation is applied in chemistry—in which the variables are food, flagellates, and waste products of flagellates, the food being converted into more flagellates and waste products. The available food material was continually decreasing as the organisms increased in number. Therefore, the autocatalytic equation may be applied to the experiments of the writer; whereas, it cannot, as previously explained, be applied to experiments of other workers with ciliates.

Richards (1928) has shown that the division rate of yeast cells in a limited volume of medium is a decreasing quantity; and furthermore, that when the medium was changed frequently, the division rate remained practically constant. Hence, neither his results nor those of the writer furnish a basis for the assumption of an autocatalyst capable of accelerating division rate.

Robertson's concept of autocatalysis in Protozoa has, of course, grown out of his numerous applications of the autocatalytic equation to growth curves of plants, of man, and of other animals. As pointed out above, *the division rate of Protozoa in cultures, as calculated from the autocatalytic equation, is an ever decreasing rather than a progressively increasing quantity.* In metazoan growth Robertson was not measuring *division rate* of cells, but rather the increase in weight (or *increase in total number of cells*) of a many-celled body. In Protozoa, on the other hand, it was the rate of cell division as well as the rate of increase of total number which he measured, and he assumed that an increase in the latter necessarily involved an increase in the former.

The rate of increase of the total number of cells in a metazoan or in a protozoan culture is accelerated during the early phase or phases of growth, but if the growth is autocatalytic, the rate of cell division is continually decreasing. In either case, the rate of increase in total number of cells (provided the increase follows the autocatalytic curve) is accelerated because the number of growing units is increasing—*not because of an acceleration* of the growth rate of the individual units, *but in spite of a decrease in the growth rate of these units.* The rate of increase of the total number of cells and the division rate of the individual cells are two distinct conceptions which should not be confused.

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THE EFFECT OF LACK OF OXYGEN ON THE SPERM AND UNFERTILIZED EGGS OF *ARBACIA PUNCTULATA*, AND ON FERTILIZATION

ETHEL BROWNE HARVEY

(*From the Washington Square College, New York University and the Marine Biological Laboratory, Woods Hole*)

It has been shown in a former paper (Harvey, 1927) that when fertilized eggs are deprived of oxygen, development is arrested, and the eggs remain in whatever phase of division they were in when oxygen was taken away; they gradually resume development and pass through subsequent phases of division when oxygen is readmitted. The experiments were performed on two species of sea-urchin occurring at Naples, *Strongylocentrotus* (*Paracentrotus*) *lividus* and *Echinus microtuberculatus*. Some of these experiments have been repeated on the Woods Hole species, *Arbacia punctulata*, and have given the same results. The present paper deals with the effect of lack of oxygen on the *unfertilized* eggs and the sperm of *Arbacia punctulata*, and on the fertilization process in these eggs. The work was done during the summer of 1929 at the Marine Biological Laboratory of Woods Hole. I wish to thank the Director for the facilities of the laboratory.

The experiments on unfertilized eggs and sperm were carried out for the most part by bubbling hydrogen through a suspension of eggs or sperm in sea-water in a closed glass vessel, from which they could be drawn off at desired intervals for observation. The connection between the hydrogen tank and the glass vessel included a quartz tube containing platinized asbestos which was kept heated to redness to remove the last traces of oxygen; from here to the glass vessel, the connection was entirely of metal and glass, sealed with De Khotinsky cement, to avoid the leakage of air which takes place through rubber connections. The length of time for complete removal of air and replacement by hydrogen, of course, depends on size of vessel, amount of sea-water, rate of bubbling, etc., but under the conditions of the experiments it required approximately twenty minutes. That a state of complete anaerobiosis obtained was shown by the fact that under similar conditions the luminescence of luminous bacteria was stopped, as ascertained by E. N. Harvey.

When unfertilized eggs are thus kept without oxygen, they are very little affected. During a period of exposure of 8 hours, one can observe no difference in appearance between the eggs when drawn from the hydrogen chamber and the control unfertilized eggs; and the exposed eggs can be fertilized and develop normally. For the first 3 hours, the eggs when withdrawn from the hydrogen chamber can be fertilized with as much ease and as rapidly as eggs kept in air; the fertilization membrane comes off at the same time (1–2 minutes) and the first cleavage plane comes in at exactly the same time (about 50 minutes) as in the control lots. When, however, eggs which have been exposed over 3 hours to hydrogen are withdrawn and fertilized, there is a slight lag (½–2 ½ minutes) in the formation of the fertilization membrane, a tendency of the membrane to adhere to the egg, a slight crenulation of the egg surface, and a lag of from 2 to 5 minutes in occurrence of the first cleavage. This was not due to the bubbling, for when air in place of hydrogen was bubbled for the same length of time through the same amount and concentration of eggs, these eggs when fertilized showed no lag in the formation of the fertilization membrane nor in time of cleavage over eggs kept at the same time undisturbed in watch glasses and fertilized. When eggs, which have been kept in hydrogen for three or more hours, are withdrawn and left in air unfertilized for 45 minutes and are then fertilized, they show no lag in membrane formation or in time of cleavage. The lag evidently represents the recovery time from exposure to the oxygen-free atmosphere. The unfertilized eggs have therefore a very short recovery period after a prolonged exposure to hydrogen, and recover instantly after a shorter exposure. They are thus in marked contrast to fertilized eggs, which require a comparatively long period (½ hour to 1 hour) for recovery from exposure to hydrogen before resuming development. It may be that the longer recovery period of the fertilized eggs from the effects of lack of oxygen is related to their greater oxygen consumption as compared with that of the unfertilized eggs. After exposure for 6 or 8 hours to either hydrogen or air (in the apparatus used) some of the eggs become cytolyzed, owing probably to the mechanical disturbance of the bubbling; the effect increases with time until, after about ten hours, practically all the eggs are cytolyzed. Whether, therefore, the life of the unfertilized egg is prolonged by lack of oxygen could not be determined by these experiments. Loeb and Lewis (1902) found that unfertilized eggs would live somewhat longer in absence of oxygen (64 hours) than in air (48 hours), and very much longer in a weak concentration (N/1000) of KCN (112 hours). This latter effect may, however, be due to destruction of harmful bacteria by the KCN as pointed out by Gorham and Tower (1902).

For experiments on sperm cells of *Arbacia,* a fairly concentrated suspension was used, one drop of fresh undiluted sperm to 10 cc. of sea-water (*i.e.,* about .6 per cent), giving a decidedly milky appearance. In such a concentration sperm live longer and retain their fertilizing power for a longer time than in a more dilute suspension, probably owing to CO_2 production as shown by Cohn (1918). When hydrogen is bubbled through the sperm suspension for about two hours, the sperm are motile immediately on withdrawal from the hydrogen chamber, or at least as quickly as they can be observed under the microscope. The lots of eggs into which they are immediately drawn form fertilization membranes and cleave at the same time as the controls. After an exposure of 2 to 3 hours, the sperm recover motility within a few seconds and fertilize eggs with a very slight lag over the controls. After an exposure of more than 3 hours, some of the sperm do not recover motility and only a fraction of the eggs to which they are added are fertilized. After 4 hours, the sperm are all inactive, do not fertilize the eggs and never recover. A control experiment in which air in place of hydrogen was bubbled through a similar amount and concentration of sperm showed that the deleterious effect is due to lack of oxygen and not to the mechanical agitation, since these sperm were just as active and potent for fertilization even after 9 hours of bubbling as are fresh sperm. It is interesting to note that the prevention of oxidations by means of a hydrogen atmosphere gives a different result from that obtained by the use of cyanides. Drzewina and Bohn (1912) found that the sperm of *Stronglyocentrotus* would survive and remain potent for 48 hours in KCN (1 : 1,000,000), and that when they were subjected to KCN for long periods (1 to 10 hours) they caused a more normal development of eggs than when subjected for a short period (30 minutes to 1 hour). Cohn (1918) found that KCN rendered *Arbacia* sperm inactive and prolonged their life, and in fact suggested that " whatever decreases the activity increases the length of their life." This is certainly not true for hydrogen. It may be, however, that some other factor associated with the absence of oxygen, such as the lack also of CO_2 is responsible for the death of the sperm in my experiments.

A study was made of individual sperm cells in the absence of oxygen by using a modified Engelmann chamber to which hydrogen was admitted and the sperm kept in a hanging drop (see Harvey, 1927). It was found that in many cases enough oxygen leaked through the vaseline seal with which the cover was mounted on the chamber to enable the sperm to keep their motility for several hours. By entangling the sperm in platinized asbestos threads, it was possible in some cases to keep them absolutely oxygen-free, and they became motionless within a half hour. If air was then admitted, the sperm immediately became motile. Even if the bubbling of hydrogen was stopped, within a very few minutes the sperm became active. It apparently requires a very minute amount of oxygen for motility of the sperm. When sperm are kept in an Engelmann chamber without oxygen for two hours, they do not recover motility on admission of air. They are killed by the absence of oxygen even more quickly than when the experiments are done in bulk.

The most interesting question in connection with lack of oxygen on eggs and sperm is whether fertilization can take place and the fertilization membrane be thrown off during complete absence of oxygen. An attempt to answer the question was made by keeping unfertilized eggs in one drop and sperm in another drop very close together in an Engelmann chamber. Hydrogen was sent through for a half hour, then the chamber was shaken so as to make the drops coalesce and the sperm come in contact with the eggs, still keeping hydrogen passing through the chamber and the seal intact. It was found that when the sperm are completely immotile, they do not fertilize the eggs, probably because they cannot get to the surface of the egg; they go in currents around and past the eggs; in no case is a fertilization membrane thrown off. On admission of air the sperm become motile and the membranes of the eggs are thrown off in 1 to 2 minutes as normally. If there is the slightest trace of air leaking in the chamber, sufficient for a few only of the sperm to be very slightly motile, some of the eggs are fertilized on mixing the drops, and fertilization membranes are thrown off, but no further development takes place until more air is admitted. The question, therefore, whether oxygen is necessary for membrane formation has not been answered. If there is absolutely no oxygen, the sperm are absolutely immotile and cannot fertilize the eggs, probably owing to mechanical difficulties, and no membranes are given off. Loeb also found that if the sperm cells of *Strongylocentrotus* were made immotile by NaCN, they were unable to fertilize the eggs even when squirted on eggs with jelly removed. If in my experiments, there is the slightest trace of oxygen, a few sperm remain motile and fertilize eggs which throw off membranes. If membrane formation does require oxygen, it is in an almost infinitesimal amount. It requires more oxygen for the development of fertilized eggs than it does for motility of sperm, fertilization of the egg and the formation of the fertilization membrane.

SUMMARY

1. Unfertilized eggs of *Arbacia* are not visibly affected by complete lack of oxygen for a period of 8 hours. After an exposure of 3 hours they recover immediately on admission of air; after a longer exposure, when air is readmitted and the eggs are fertilized, there is a slight lag in the formation of the fertilization membrane and in time of cleavage.

2. Sperm of *Arbacia* are rendered motionless by lack of oxygen, but are otherwise unaffected for 2 hours. They recover immediately on admission of air. After 3 hours some of the sperm are irreversibly injured, and after 4 hours they are all killed.

3. When sperm are added to unfertilized eggs, both being in complete absence of oxygen, fertilization does not take place, and the fertilization membrane is not thrown off because the sperm are not motile, and cannot get to the surface of the egg. The membrane is thrown off immediately on admission of air. If there is the slightest trace of air, which may leak through the vaseline seal to the chamber, sufficient for only a few sperm to be very slightly motile, the eggs with which they come in contact throw off fertilization membranes, but do not develop further until more air is admitted. If oxygen is necessary for membrane formation, it is in an almost infinitesimal amount.

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THE EFFECT OF CONJUGATION WITHIN A CLONE OF PARAMECIUM AURELIA

DANIEL RAFFEL

(From the Zoölogical Laboratory of the Johns Hopkins University)

Introduction

On the effects of conjugation in paramecium, particularly in relation to the production of inherited variations, the results of investigators are in conflict. Jennings (1913), working with both *Paramecium aurelia* and *Paramecium caudatum,* reported that conjugation increased inherited variations: that it caused the production of diverse biotypes. The members of a clone—a population derived by fission from a single individual, whether an ex-conjugant or not—remained nearly or quite uniform in their inherited characteristics so long as conjugation did not occur among them. But after conjugation within such a clone, the inherited characteristics of descendants of the different ex-conjugants had become diverse. Thus by conjugation many different biotypes had been produced, the descendants of each ex-conjugant constituting a single uniform biotype.

Calkins and Gregory (1913), on the other hand, reported that there is in *Paramecium caudatum* as much variation among the descendants of the four individuals produced by the first two fissions of a single ex-conjugant as was found between the progeny of different ex-conjugants. They conclude that, " The results of this study show that physiological and morphological variations in the progeny of a single ex-conjugant of *Paramecium caudatum* are fully as extensive as the variation between the progenies from different ex-conjugants" (p. 523).

Jennings (1916, p. 528, and 1929, p. 188) has tried to show that the results of Calkins and Gregory are invalidated by uncontrolled sources of error. On the one hand, he holds that their method of culture permitted continuing environmental differences between their different populations, such as would give rise to differences that would appear to be hereditary, although they were not. On the other hand, he notes the occurrence of conjugation within some of their cultures and the fact that this might readily have occurred undetected. This would vitiate their conclusions.

Obviously, the situation calls for a new investigation of the matter, in which such methods shall be employed as shall certainly exclude the possibility that environmental differences affect the results, while at the same time the occurrence of unobserved conjugation is excluded. It is such an investigation that is here presented. In order to assure a uniform environment for all the lines of descent an elaborate technique was employed. This is described on later pages. The method involved, first, the use of a synthetic culture medium of known composition, with pure cultures of food organisms and uniform glassware; second, continuation of the uniform conditions by the cultivation of the paramecia under aseptic conditions; third, frequent testing of the culture fluid in which the organisms have lived in order to ascertain whether the uniformity of the environment has been maintained. In addition, the organisms are cultured singly and transferred daily to new drops of culture fluid, so that it is impossible for conjugation to occur. Continuing diversities between lines cultivated simultaneously under such conditions can be interpreted only as caused by constitutional differences among the organisms, not as due to diversities in food or cultural conditions, or other extrinsic factors.

Taking these precautions, two comparisons are made. First, a population descended from different ex-conjugants is compared with a population derived by fission from non-conjugants of the parent clone. Second, four lines descended from each ex-conjugant are compared with one another, and the several such different clones are similarly compared. In this way it is possible to determine whether increased hereditary variation and differentiation into diverse biotypes are produced by conjugation.

The investigation was suggested to me by Professor H. S. Jennings, and my thanks are due to him for assistance throughout the work. I am also indebted to Rose Mahr Raffel, who assisted in the carrying out of the experiment, and without whose aid cultures of this magnitude, using the elaborate technique here employed, could not have been carried through.

Materials and Methods

In this investigation an elaborate technique was used in order to subject all of the lines to identical environmental conditions. Great care was taken to eliminate any possible sources of variation. To this end the culture fluid, the food supply and the glassware used were standardized to as great an extent as was possible. The work which has been carried on for several years by Hartmann and his associates at the Kaiser-Wilhelm Institut für Biologie has made possible the use of synthetic culture fluids and pure cultures of food organisms for the cultivation of protozoa. The use of pure cultures of unicellular algæ as food organisms appears first in the work of Luntz (1926) on the rotifer *Pterodina elliptica* and more recently in the work of Adolph (1929) with the ciliate *Colpoda*. The results of the work of Hartmann and his associates are given in a recent paper of Belar (1928). The following pages contain a detailed account of the methods used to obtain uniformity in the environmental conditions throughout this experiment.

1. *Culture Fluid*

The culture medium used was a physiological salt solution of known composition. After many attempts to find a solution in which the race of *Paramecium aurelia* which was used would live, it was found that if the solution described by Pringsheim (1928) for the cultivation of algæ was altered so as to be neutral, it furnished an excellent medium for this organism. This modification was obtained by replacing the KH_2PO_4 used by Pringsheim by an equal molar concentration of K_2HPO_4. The composition of the solution was KNO_3 0.5 gram, K_2HPO_4 0.06 gram, $MgSO_4$ 0.02 gram, $FeCL_3$ 0.001 gram, water 1000 grams. The water used in making this solution was redistilled from a still made of Pyrex glass and had in all cases a conductivity less than 1.05×10^{-6} mho. This solution was made up in quantities of one liter. It was then divided into portions of approximately 15 cc. in test tubes. These test tubes were plugged with non-absorbent cotton and the solution was sterilized in the autoclave for 15 minutes under 15 pounds of steam pressure. The solution was kept in this way for periods varying from a few days to two months before it was used. Tubes tested at intervals showed no bacteria and no measurable alterations in composition.

2. *Food Organism*

The food organism used was a unicellular green alga, *Stichococcus bacillaris*.[1] This was cultivated on 0.05 per cent Benecke's agar composed of water, 1000 grams; Agar-Agar, 15 grams; NH_4NO_3, 0.2 gram; $CaCl_2$, 0.1 gram; $MgSO_4.7H_2O$, 0.1 gram; and K_2HPO_4, 0.1 gram. The components of this agar were boiled together until the agar-agar was all dissolved. Five cc. portions were poured into test-tubes which were then sterilized in the autoclave under 15 pounds of steam pressure for fifteen minutes. These tubes were then "slanted" in order to obtain a large, easily accessible surface. Twenty of these tubes were seeded from a pure culture of the alga on successive days. After this the slants were used in the order in which they had been seeded and as they were used they were replaced by new tubes seeded from them. The tubes in which the alga was cultivated were kept constantly before a north window in order to obtain sufficient light.

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[1] I am indebted to Professor W. R. Taylor of the University of Pennsylvania for the identification of this organism.

Each day the tube of *Stichococcus* to be used that day and a fresh tube of the culture fluid were opened close to a flame into which their open ends were immediately thrust. Then a small quantity, approximately 5 cm., was scraped from the agar with a platinum loop which had just been sterilized in the flame. This small quantity of the alga was then quickly suspended in the solution and both tubes were immediately restoppered. Then a new tube of agar was seeded from the same tube and replaced in its proper place in the rack. Many tests of the suspension were made from time to time and in no case was any bacterial contamination found. An effort was made to have the suspension of alga in the solution always of the same density. However, no method more accurate than a comparison of the appearance of the tubes was found for determining the success of this effort. For this reason, preliminary experiments were performed in order to determine whether or not the quantity of algæ used affected the rate of reproduction of the paramecia. It was found that sufficient algæ to produce a slight greenish tinge in the suspension furnished enough food for these organisms. Greater densities than this had no effect on the rate of reproduction even when they were far in excess of any used in the actual experimental work. At all times an excess of algæ was assured and the drops containing the paramecia always showed a large number of the algæ at the end of the period during which the organisms remained in them.

It was found, however, that if the paramecia were kept in this solution with this single food organism, they were unable to live and reproduce. If a very slight trace of a *Bacillus candicans* was present, this difficulty was eliminated.[2] Attempts were made to cultivate the paramecia on this bacillus in the absence of the alga. All such attempts failed, and when a mixture of the two food organisms was used, the food vacuoles were dark green in color—indicating that the food supply was composed mainly of the alga. After a slight trace of this bacterium was once introduced into a culture of paramecium, it was perpetuated in the transfers of the organisms. As far as it was possible to determine by plating in the usual way, this bacterium was present in approximately the same quantity from day to day in all of the many cases tested at random. It was thought advisable, however, to determine whether or not differences in the quantity of this organism present affected the rate of fission of the paramecia. There was no difference in the effect produced by the presence of any quantity of the bacterium less than that required to make the drops of culture fluid appear milky. At no time during the course of this investigation was this condition approached.

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[2] I am indebted to Professor W. W. Ford, Professor of Bacteriology in the School of Hygiene and Public Health of the Johns Hopkins University, for the identification of this bacillus.

3. *Glassware*

The various lines of paramecium used in this investigation were cultivated on slides with two concavities. It was found from preliminary work that different slides affected the paramecia differently. On some slides representatives of all the lines tested reproduced more rapidly than did other representatives of the same lines on other slides. The pH of drops of culture fluid which had remained on the different slides was tested and was found to vary greatly. Drops of fluid which had been identical when placed on the slides were found to vary by a whole pH unit within twenty-four hours. This showed that the glass of the various slides differs in solubility. New slides were then obtained, all of the same kind of glass. These slides were of French origin. After two days the organisms grown on these slides died out. No amount of washing the slides with various kinds of solvents made it possible to cultivate organisms on them. Investigation disclosed that this French glass is made by a process involving the use of lead. It appears that the presence of this element was responsible for the toxic effects of these slides on the organisms. When this was discovered, new slides were obtained which were of white glass and were all produced by the same manufacturer. These slides were the only ones used in this investigation. Before they were used they were thoroughly washed in running water. Then they were washed in ether and 95 per cent alcohol in order to remove any organic matter with which they might have been contaminated. They were again thoroughly washed with running water, rinsed in several changes of tap water and finally rinsed in hot distilled water. Each day the slides were thoroughly washed in the following manner. First they were held, individually, in running tap water and the depressions were rubbed well with the thumb. They were then placed in a receptacle containing clean tap water. In this receptacle they were rinsed three times. Then, after the last tap water was thoroughly drained off, the slides were covered with hot distilled water. They were then dried on racks.

In order to prevent contamination of the cultures by bacteria in the air, Petri dishes 100 mm. in diameter and 15 mm. deep were used as moist chambers. This made it possible to transfer the organisms with a minimum of exposure to the air. The dishes contained water at the bottom; the two slides to each dish were supported above this on strips of glass. After the Petri dishes, the slides, and the glass plates were assembled, they were heated in the hot air sterilizer for one hour at 150° C. In order to facilitate the handling of the numerous dishes which were used, baskets were made from ¼ inch wire netting which held a dozen Petri dishes in four tiers of three dishes each.

The organisms were transferred by means of capillary pipettes. Each of these contained a plug of cotton inserted into its wide end. This is a precaution necessary to prevent contamination of the cultures by microörganisms which would otherwise be introduced by the rubber bulbs used on the ends of the pipettes. The glass part of the pipettes with their cotton plugs were kept in large museum jars, in which they were heated in the hot air sterilizer for one hour at 150° C. before each time they were used.

4. *Method of Transferring Organisms*

Before the daily transfers were made, the Petri dishes were removed from the hot air sterilizer. Then two drops of the culture suspension were dropped into each concavity. Large pipettes which were drawn out until the ends were 2 mm. in diameter were used for this purpose. These pipettes, like the ones used for transferring the paramecia, were protected by cotton plugs and were sterilized before each time that they were used. The mouth of the test tube containing the suspension of culture fluid was sterilized in the Bunsen flame each time that it was opened. The tops of the successive Petri dishes were then raised on one side, the pipette was introduced and two drops were allowed to fall into each concavity. Four dozen dishes were prepared in this manner at one time. From time to time bacteriological plates were prepared from culture medium which was treated in the manner described above, after it was left for twenty-four hours. In every case the plates were negative, thus indicating that the technique was absolutely dependable.

In transferring the animals a Petri dish containing the two slides was placed on the stage of the binocular microscope. Another dish containing new culture fluid was placed at the experimenter's right. One organism was then taken from each concavity and transferred to the corresponding concavity of the new dish. This was done very rapidly, using a clean pipette that had just been removed from the jar of sterile pipettes. A separate pipette was used for the organisms of each dish. The new dishes were then removed to the constant temperature chamber, in which they were left at a temperature of approximately 24° C. (There was in the history of the cultures variation in temperature from 22.2°–26.2° C.)

5. *Isolation and Sterilization of the Clone*

The various lines of *Paramecium aurelia* used in this investigation are the descendants of a single individual which was isolated from a mass culture in the laboratory on July 29, 1929.

Parpart (1928) has shown that spores of bacteria may be, and often are, carried within paramecium and that in washing these organisms, precaution must be taken to eliminate these spores as well as the bacteria external to the paramecium. For this reason, when the individual which was used to start the clone for this investigation was washed, the precautions suggested by Parpart were observed. The individual was first washed successively in five concavities containing sterile culture fluid. Then at intervals of one hour it was washed through five more similar quantities of fresh culture fluid. It was then placed in a concavity containing the regular culture suspension described above in which there was a slight trace of the *Bacillus candicans.* No bacteria were added at any later time. From time to time throughout the course of the experiment bacteriological plates were made from drops from which the paramecia had been removed. Several dishes containing both ex-conjugant and non-conjugant lines were taken at random for this purpose. At no time did any plate made in this way indicate the presence of any bacteria except the bacterium which had been introduced at the beginning.

The Experiment

1. *Plan*

The plan of the experiment was as follows: A clone was obtained by allowing a single individual of *Paramecium aurelia* to multiply. A portion of the clone was induced to conjugate, while another portion was kept without conjugation. The former, after the separation of the pairs, yields lines of descent that constitute the ex-conjugant population, the latter the non-conjugant population. These two populations are later compared as to their mortality, fission rate, variation and the inheritance of the variations.

For comparison with the results of Calkins and Gregory, a method similar to theirs was employed for the grouping and subdivision of the ex-conjugant lines. Each of the two members of a pair was allowed after separation to divide twice, yielding four individuals of common origin, the four *quadrants*. From each quadrant a line of descent was obtained. Each set of four quadrants derived from a single ex-conjugant is called, for convenience, a *tetrad*. The variation within single tetrads is compared with the variation among lines belonging to different tetrads (and so derived from different ex-conjugants). This tests whether the diversity among the descendants of a single ex-conjugant is as great as that between those of different ex-conjugants (as is maintained by Calkins and Gregory).

2. *Description*

The experiment was begun with the isolation of a single organism on July 29, 1929. The progeny of this individual were propagated on slides by daily transfer until August 5, 1929. By this time there were approximately 1500 individuals present. On the morning of August 5, all of the individuals, except one, from each concavity, were transferred to two small sterile culture dishes contained within Petri dishes. No fresh culture fluid was added to those culture dishes and the least possible quantity was carried over with the organisms. The other organisms were transferred to clean slides in the usual manner. These latter ones were the source from which the non-conjugant lines used in this experiment were obtained. The process of transferring this number of animals occupied several hours. Before all the organisms had been transferred conjugation had begun in the two culture dishes. One hundred and twenty pairs of conjugants were removed from the culture dishes and numbered in order of their removal. The next morning the pairs had separated. The two members of each pair were transferred to the two concavities of a clean slide. The non-conjugants, one from each dish which had been transferred to slides on August 5, were transferred to clean slides until 112 non-conjugants had been transferred. The number of fissions was recorded in the case of the non-conjugants. On August 7–8 the ex-conjugants completed their first two divisions, giving rise to the four lines or quadrants from each of the ex-conjugants which were to be propagated in this experiment.

On August 7th and 8th the non-conjugant lines and the ex-conjugant lines were so distributed that no two non-conjugant lines or lines from the same tetrad were cultivated in the same Petri dish. This was done so that if any correlation was found between the quadrants of a tetrad or between lines of the non-conjugant population, it could not be the result of cultivation on the same slides or in the same dishes.

From August 6th to September 10th inclusive, each line was transferred each day (except on August 8th and 10th as described below). On August 8th and August 10th the amount of work was so great that it was not possible to complete the transferring until after midnight. The lines which were not transferred until after midnight on these days were not transferred again for approximately 36 hours. On August 11th all of the lines which were incomplete because of losses were discarded. When this was done, the number of lines retained was the maximum number that two persons could transfer once daily, using this involved technique. From August 11th to the close of the experiment on September 10th, all the lines surviving were transferred daily.

The actual numbers isolated at the beginning of the experiment were for the non-conjugants 112; for the ex-conjugants 405 lines or "quadrants" derived from 105 different ex-conjugants, belonging to 58 different pairs. The numbers were reduced by accident or death of lines, so that the actual numbers of lines available for comparison were, for the first ten days of the experiment, 66 non-conjugants, 324 ex-conjugants; for the first twenty days, 64 non-conjugants, 295 ex-conjugants; for the entire period of 36 days, 46 non-conjugants, 115 ex-conjugants.

During the first week following the beginning of the experiment a rather large number of deaths occurred among the non-conjugant lines. After this period there occurred a period of about three weeks during which deaths among the non-conjugant lines were rare. Many of the deaths which occurred during the early part of this period were lines that had stopped dividing during the earlier period. On the twenty-fifth day of the experiment (August 29th), the rate of mortality among the ex-conjugant lines increased rapidly. This was followed two days later by an increase in the rate of mortality among the non-conjugants. This high rate of mortality continued for nearly ten days. The occurrence of this high rate of mortality in the ex-conjugant lines beginning twenty-five days after conjugation was accompanied by a general depression in all the lines. This fact and the occurrence of two such periods in the non-conjugant lines, separated by a period of about twenty-five days, led to the suspicion that these were periods of endomixis. On September 6th many of the excess animals from the non-conjugant and ex-conjugant lines were stained and mounted for study. The individuals from the ex-conjugant lines showed in many cases the conditions of late stages of endomixis. Numerous fragments of macronuclei were present, and in one case the organism was found to be at the climax of the endomictic process. The representatives of the non-conjugant lines showed on the whole the conditions typical of earlier stages of endomixis. Large irregular macronuclei were found, often accompanied by large fragments. It seems clear, therefore, that the periods of high mortality were periods of endomixis; a relation which other investigators have observed.

On September 10th, thirty-six days after conjugation had occurred, the experiment was discontinued. At this time 46 lines of non-conjugants and 115 lines of ex-conjugants were still in existence.

3. *Results*

The experiment was designed to supply data mainly upon the rate of reproduction, its variability and the inheritance of the variations, in the ex-conjugants and non-conjugants. It yields also certain data on comparative mortality, which will be given first.

A. Mortality

A considerable number of the lines of both the non-conjugants and ex-conjugants died out during the thirty-six days of culture. The percentages surviving in each group at certain periods after the beginning of the experiment, are the following:

After	20 days	25 days	35 days
Non-conjugant lines......	73.0	68.6	51.7
Ex-conjugant lines.......	79.2	67.9	30.8

Thus on the whole the mortality is much greater among the lines descended from the ex-conjugants. At the end 51.7 per cent of the non-conjugant lines were alive as against 30.8 per cent of the ex-conjugant lines.

B. Rate of Reproduction

The basic data as to comparative rate of reproduction in the non-conjugants and ex-conjugants are given in Table I. The number of fissions for both groups is reckoned from August 7th, on which day all of the ex-conjugants divided once or twice. Thus the statistics are not affected by any delay in fission due to the process of conjugation itself.

Table I shows at *A* the number of fissions for the different lines for the 20 days of culture beginning August 7th and ending August 26th; throughout this period there were 64 lines of non-conjugants and 295 lines of ex-conjugants, the latter derived from 99 different ex-conjugants and so forming 99 tetrads. At *B* are shown the distribution

TABLE I

Distributions of the total numbers of fissions and the daily fission rates, for the different lines cultivated, for different periods of the experiment. The mean fissions and fission rates are also given (for certain periods), for the different tetrads (total lines derived from a single ex-conjugant).

A. Fissions for the First 20 Days, Aug .7 to 26 inclusive

	Number of Fissions in 20 Days																						Total Lines	Mean Fissions
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37		
Non-conjugants........													2	1	5	2	17	12	14	6	4	1	64	32.9 ± 0.2
Ex-conjugants.........	1		1			1	1	1	2	3	5	7	12	27	30	55	43	45	36	14	10	1	295	31.3 ± 0.1
Means of tetrads.......		1				1				1	1	2	3	5	15	17	20	19	11	3			99	31.2 ± 0.2

B. Fissions for the 35 Days, Aug. 7 to Sept. 10 inclusive

	Number of Fissions in 35 Days																								Total Lines	Mean Fissions
	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61		
Non-conjugants........										1	1			4	1	2	2	4	5	10	6	5	3	2	46	56.1 ± 0.3
Ex-conjugants.........	2		1	1	1		1	1	2	3	2	2	5	9	5	13	18	16	9	6	7	6	3	2	115	53.3 ± 0.3

C. Mean Daily Fission Rates, for the Period Which Each Line Lived, for All Lines That Lived More Than 10 Days

	Mean Daily Fissions																					Total Lines	Mean Daily Rate
	.85	.90	.95	1.00	1.05	1.10	1.15	1.20	1.25	1.30	1.35	1.40	1.45	1.50	1.55	1.60	1.65	1.70	1.75	1.80	1.85		
Non-conjugants........									1		4	3	7	2	8	15	13	10	3			66	1.58±0.01
Ex-conjugants.........	1	2		1	1	2	4	7	15	18	20	24	36	50	71	29	24	14	4		1	324	1.48±0.01
Tetrads............		1			1	1		1	3	1	6	11	22	19	16	12	7					101	1.47±0.01

of the numbers of fissions for the 46 surviving non-conjugant and 115 ex-conjugant lines that survived throughout the entire period of 36 days, August 6th to September 10th. At *C* in Table I are shown the mean fission rates for the total period of survival, for all the lines that lived more than 10 days.

Mean Rate.—As Table I shows, the mean rate of reproduction for the non-conjugant and conjugant groups did not differ greatly, although in every case the mean rate for the non-conjugants is higher by a small but significant amount. The mean fission rate for all non-conjugant lines is 1.58 ± 0.01; for all ex-conjugant lines, 1.48 ± 0.01.

C. Variation in Fission Rate

But it is in the variation of the fission rate that the difference between the non-conjugants and ex-conjugants is striking. An inspection of Table I shows at once that the variation in the ex-conjugant lines is much greater than that in the non-conjugant lines. For the first twenty days, the number of fissions in the non-conjugant lines varies from 28 to 37, a range of 10. For the ex-conjugant lines, in the same period the range is from 16 to 37, a range of 22, more than double that for the non-conjugants. For the entire 35 days, the non-conjugant lines range from 47 to 61, the ex-conjugant lines from 38 to 61. The mean daily fission rates (*C*, Table I) vary in the non-conjugants from 1.25 to 1.75; in the ex-conjugants from 0.85 to 1.85. The range for the former is 0.55; for the latter 1.05, or nearly double that for the non-conjugants. The fission rate for the lowest lines of ex-conjugants is far below that for the lowest non-conjugants, and the highest ex-conjugant line is above the highest non-conjugant. Conjugation within the clone has caused a wide spreading out of the fission rates; it has produced stocks with lower, and with higher, rates than any found in the clone before it has conjugated.

Computation of the standard deviations and coefficients of variation shows the same great increase in variation as a consequence of conjugation. The means, standard deviations and coefficients of variation, computed from the data shown in Table I, are given in Table II.

As Table II shows, the coefficient of variation of the ex-conjugant lines is for the first 20 days 158 per cent of that of the non-conjugants; for the entire 35 days it is 187 per cent of that of the non-conjugants. For the mean daily fission rates of the different lines, the coefficient of variation for the ex-conjugants (10.14) is 139 per cent of that of the non-conjugants (7.28).

The comparative distribution of the fission rates of non-conjugants and ex-conjugants, as shown in Table I, are worthy of notice. In the first 20 days (*A*. Table I) 22 lines of ex-conjugants, or 7.4 per cent of all, show fewer fissions than any of the non-conjugants. In the entire 35 days (*B*, Table I), the proportion is nearly the same: 7.8 per cent of the ex-conjugant lines lie below all of the non-conjugant lines. At the opposite extreme the two sets are alike; the highest lines have the same number of fissions in the two cases. In mean daily fission rates, 18 lines of ex-conjugants, or 5.6 per cent of all, lie below all of the non-conjugant lines, while one ex-conjugant line lies above all the non-conjugant lines.

Table II

Means, standard deviations and coefficients of variation of non-conjugant and ex-conjugant lines, for the numbers of fissions during certain periods; and for the mean daily fission rates of the different lines. Based on the data given in Table I.

A. Numbers of Fissions

	First 20 Days			Total 35 Days		
	Mean	Stan. Dev.	Coef. Var.	Mean	Stan. Dev.	Coef. Var.
Non-conjug'ts	32.9±0.2	1.88±0.11	5.70±0.34	56.1±0.3	3.19±0.22	5.68±0.40
Ex-conjugants	31.3±0.1	2.83±0.08	9.02±0.25	53.3±0.3	4.49±0.20	8.42±0.38

B. Mean Daily Fission Rates of the Different Lines

	Mean	Stan. Dev.	Coef. Var.
Non-conjugants...	1.58 ± 0.01	0.12 ± 0.01	7.28 ± 0.43
Ex-conjugants....	1.48 ± 0.01	0.15 ± 0.00	10.14 ± 0.27

It is clear, therefore, that conjugation within the clone has much increased the variability of the fission rate, and that one of the factors in the increased variability is the production of a considerable number of ex-conjugant lines that have a lower fission rate than any lines among the non-conjugants.

D. Variation among Quadrants Derived from a Single Ex-conjugant, Compared with Variation Between Lines Derived from Different Ex-conjugants

Calkins and Gregory (1913) reached the conclusion that the variation between different quadrants (the four lines derived from a single ex-conjugant) was as great as that between lines derived from diverse ex-conjugants. Lines derived from a single ex-conjugant constitute, of course, a clone within which conjugation has not occurred; so that according to this result, there is no increase of variation in consequence of conjugation within the clone. To test this particular matter, the variation between the different quadrants of the same tetrads (each tetrad derived from a single ex-conjugant) was compared with the variation among progeny of the different ex-conjugants. For each tetrad records of only two to four lines are available, so that the coefficients of variation within the tetrad are not statistically adequate, but the general result is of interest. For the number of fissions during the first 20 days of the experiment, the mean coefficient of variation for the lines constituting a single tetrad was 4.53; for the means of the diverse tetrads (progeny of the diverse ex-conjugants), the coefficient of variation was 8.32. For the average daily fission rate, the mean coefficient of variation for the lines constituting a single tetrad was 5.22; for the diverse tetrads it was 8.57.

As will be seen by comparison with Table II, the mean variation within tetrads (4.53) is of a similar order to the mean variation for non-conjugant lines of a clone (5.70) (that is, to the variation within a clone in which conjugation has not occurred). On the other hand, the variation when the different tetrads are compared (8.32) is much greater, and is similar to the variation (9.02) when all the lines derived from ex-conjugants are compared. This indicates strongly that the four quadrants produced by the first two divisions of an ex-conjugant do not differ in any general way from any other products of fission of a single individual. Further, the similarity between the coefficients of variation for all ex-conjugant lines taken separately, and that for the means of the diverse tetrads, indicates that the variation among the ex-conjugant lines is due mainly to the inherent differences between the ex-conjugants.

The higher variation among diverse tetrads, as compared with less variation between the quadrants belonging to the same tetrads, may be further shown by comparing the maximum differences found (1) between any two lines of the original non-conjugant population; (2) between quadrants belonging to a single tetrad; (3) between the means of diverse tetrads; and (4) between any two ex-conjugant lines. These comparisons are given in Table III.

This table shows that the maximum difference within any of the 99 tetrads and the maximum difference between any two non-conjugant lines of the original population are very nearly identical. On the other hand, the maximum differences between any two ex-conjugant lines are only slightly greater than the maximum differences between any two of the tetrads. (It is to be expected that the maximum differences between two tetrads would be slightly smaller than that between the two ex-conjugant lines which differ most, since the fissions for tetrads are usually the means of two to four lines.) Thus the single tetrads do not significantly differ in their variability from the general non-conjugant population, while the variation between the different tetrads is much greater than that within the tetrads.

Table III

Maximum Differences Between Lines Having Different Relations to Each Other with Respect to Conjugation

	Total Fissions First 20 Days	Average Daily Fission Rate
Maximum difference between two non-conjugant lines of the original population . . .	9	0.50
Maximum difference within any tetrad	10	0.58
Maximum difference between two means of tetrads	17.75	0.84
Maximum difference between two ex-conjugant lines	21	1.00

The matter may be tested further by determining whether there is correlation in fission rates between the members of the tetrads. If the different quadrants within the tetrads differ as much as do the members of different tetrads, there should be no correlation between the members of the tetrads. If, on the other hand, the different quadrants of the single tetrads show a significant correlation, this will demonstrate that such quadrants are not so unlike as are different lines of the ex-conjugant population taken at random.

The data for this comparison are shown in Table IV, based on the numbers of fissions during the first 20 days of the experiment. The fissions for each quadrant of each tetrad are entered as X against the fissions for each other quadrant of that same tetrad as Y. As some of the tetrads had but two surviving lines, others three or four, the total number of entries in the table is 332 pairs. Since the members of the tetrads are like variates, the correlation must be computed as for a symmetrical table in which each pair is entered twice, in reverse order (see Jennings, 1911, for the method of computation).

The coefficient of correlation between the fission rates of the quadrants of the same tetrad, obtained from this table, is very high, amounting to 0.854 ± 0.007. Beyond question, therefore, the quadrants derived from a single ex-conjugant are much more alike in their fission rates than quadrants derived from diverse ex-conjugants.

All the four lines of evidence thus agree in showing clearly that a population composed of different ex-conjugants of a clone has a higher variation in fission rate than do the offspring of single ex-conjugants. (1) The coefficient of variation is much higher for the ex-conjugant population than for the non-conjugant. (2) The coefficients of variation for quadrants belonging to single tetrads is much less than the coefficient of variation for the means of diverse tetrads. (3) The maximum differences between lines within tetrads are much less than the maximum differences between means of different tetrads. (4) There is a very high correlation (0.854) between the lines or quadrants derived from the same ex-conjugant. These four lines of evidence establish firmly the fact that conjugation within a clone causes increase of variation.

Table IV

Paramecium aurelia. Correlation between total number of fissions, August 7–26, of each member of the tetrad with every other member.

	18	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
16	1																	1
22			1							1								2
23										1								1
24		1	1							1								3
25						2												2
26						1				1								2
27					1	3				2	1							7
28				2	2			1	1	3	2	1				1		13
29						1		1	2	3	6	7	3	2	3			28
30					1	2			6	6	8	6	3	1	1	1		35
31					1		4	2	5	7	14	5	5	7	3	4		57
32					1	1		4	5	6	7	7	10	5	2	1		49
33							1	1	6	8	10	11	10	10		1		58
34							1		3	1	9	3	16	6	3	1		43
35											3	7	3	4	1		1	19
36						1	1	1		1	1			4		1		10
37												1			1			2
	1	1	2	2	6	11	7	10	28	41	61	48	50	39	14	10	1	332

r = + 0.854 ± 0.007

E. Inheritance of the Diverse Fission Rates

In order to determine whether the diverse fission rates observed are hereditary, the number of fissions which occurred in each line during the first ten days after the ex-conjugants began to divide was correlated with the number of fissions during the following ten days. These coefficients of correlation were obtained (*a*) for all of the non-conjugant lines which lived from August 6th–26th, (*b*) for all of the ex-conjugant lines which lived through the same period, and (c) for the means of all the tetrads of which one or more "quadrants" survived until August 26th. The correlation tables from which these coefficients of correlation were calculated are given in Tables V, VI and VII. No correlation was found among the non-conjugant population (Table V). The coefficient of correlation obtained was + 0.016 ± 0.084. Thus the differences in fission rate (which are not very great, as Table V shows) are not inherited differences.

TABLE V

Paramecium aurelia. Non-conjugant lines. Correlation of number of fissions, August 7–16 and August 17–26.

	13	14	15	16	17	18	19	20	
13			1						1
14		1	1	2					4
15			1	1	3	3			8
16		1	1	8	6	1			17
17	1		4	2	5	2			14
18		2	2	7	3	1			15
19				1	2				3
20				1	1				2
	1	4	10	22	20	7			64

r = + 0.016 ± .084

TABLE VI

Paramecium aurelia. Ex-conjugant lines. Correlation of number of fissions, August 7–16 and August 17–26.

	6	9	10	11	12	13	14	15	16	17	18	19	
10	1			1		1							3
11				1			1			1			3
12	1					1	2		2				6
13				2		1	3	3	4	1			14
14					1	2	3	9	7	6			28
15					2	1	11	9	16	11	2	1	53
16		1	1		1	3	8	24	20	19	6		83
17				1		4	8	10	17	18	2	1	61
18					1	1	1	6	10	10	3		32
19						1	1	1	1	5	1		10
20								1	1				2
	2	1	1	5	5	15	38	63	78	71	14	2	295

r = + 0.327 ± 0.035

For all the ex-conjugant lines, each taken separately (Table VI), there is a well-defined positive correlation of + 0.327 ± 0.035. Thus many of the diversities between these are inherited. When, however, the means of the separate tetrads are taken, and the fissions for the first ten days are tabulated against those of the second ten days (Table VII), the correlation rises to + 0.651 ± 0.039. In such a table, the process of averaging the different quadrants of the tetrad smoothes out in large measure the accidental differences between the diverse lines, leaving mainly the intrinsic differences, which are inherited; hence the high coefficient of correlation.

TABLE VII

Paramecium aurelia. Means of tetrads. Correlation of number of fissions, August 7–16 and August 17–26.

	6.00	11.00	12.00	12.50	12.67	12.75	13.67	14.00	14.25	14.67	14.75	15.00	15.25	15.33	15.50	15.67	15.75	16.00	16.25	16.33	16.50	16.67	16.75	17.00	17.25	17.33	17.50	
10.50		1																										1
11.00	1																											1
12.00						1																						1
13.50				1																								1
14.00							1	1					1						1		2							6
14.25									1																			1
14.33															1													1
14.50										1						1												2
14.67			1																1									2
14.75													1															1
15.00			1								1		2		1				2				1					8
15.25													1	1			1											3
15.33								1					1			1												3
15.50													1	1		1					1						1	5
15.67					1			2											1		1							5
15.75								1					1					1								1		4
16.00								1				1	1	2				1	5			1		2				14
16.25								1					1	1				1			1							5
16.33																	1					1						2
16.50																2		2	1		1			1				7
16.67										1			1						1	1		1						5
16.75																	1						1					2
17.00												1							2		2			1				6
17.25																		1		2	1							4
17.33																1			1	1								3
17.50															1						1				1			3
17.67																			1					1			1	3
	1	1	2	1	1	1	1	7	1	2	1	2	11	5	3	6	3	6	16	4	10	3	2	5	1	1	2	99

r = + 0.651 ± 0.039

Thus in a non-conjugant population there is no indication of hereditary diversities between the different lines, while among the lines derived from different ex-conjugants, hereditary diversities are clearly present. A population of ex-conjugant lines consists of diverse biotypes produced by conjugation from a homogeneous clone.

F. Similarity Between the Lines Derived from the Two Members of a Pair of Conjugants

Jennings (1913) and Jennings and Lashley (1913) found that the lines derived from the two members of a pair of conjugants resembled each other more than do the progeny of ex-conjugants derived from different pairs. An attempt was made to determine whether this relation holds for the population studied in this investigation. This was done by correlating the mean number of fissions for twenty days, and the mean average daily fission rate of all lines which lived for more than ten days, of the two tetrads derived from each pair of conjugants. The coefficients of correlation which were obtained were $+0.102 \pm 0.073$ and -0.188 ± 0.070. Because of the small number of pairs involved and the large probable error obtained, these coefficients of correlation are of uncertain significance. Another experiment using a large number of pairs is planned, in order to investigate this matter further.

Summary and Discussion

This paper gives an account of an investigation designed to test critically the question whether conjugation produces inherited variation within a clone of *Paramecium aurelia.* An elaborate technique was devised and carried through, to prevent the occurrence of environmental diversities among the lines of descent tested: a synthetic culture fluid was employed; pure cultures of food organisms used, and the glassware standardized to the highest possible degree.

Cultivated in this way, ex-conjugant lines of descent were compared with non-conjugant lines from the same parent clone, with respect to the rates of fission. The results are:

(1) Conjugation greatly increased the variation in fission rate. The population composed of lines descended from ex-conjugants showed a much greater range of variation and a much greater coefficient of variation than did the population derived from non-conjugants. The variation was extended by conjugation mainly in the direction of lowered fission rate. A considerable proportion of the ex-conjugant lines had a lower daily fission rate than any of the non-conjugant lines. Others had as high a fission rate as the non-conjugants (see Table I).

(2) The four quadrants derived from the first two divisions of single ex-conjugants showed when compared only such variation as is

found in non-conjugants; not at all such extreme variations as are found between lines derived from diverse ex-conjugants. The four quadrants from a single ex-conjugant are highly correlated in their fission rates, showing a correlation coefficient of 0.854 ± 0.007. Such quadrants derived from a single ex-conjugant are thus much more alike in their fission rates than are lines derived from diverse ex-conjugants. There is no indication that the first two fissions occurring after conjugation have any effect in segregating diverse lines, or that they differ in their effects from any other fissions.

(3) The diverse fission rates of lines or populations derived from different ex-conjugants are in large measure inherited, while the differing rates of non-conjugant lines are not inherited.

The work therefore leads to the following conclusions: Conjugation within a clone of *Paramecium aurelia* produces diverse biotypes, having different inherited fission rates. The fissions of a single ex-conjugant do not give origin to diverse biotypes; this is as true of the first two fissions after conjugation as of later fissions.

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A MECHANISM OF INTAKE AND EXPULSION OF COLORED FLUIDS BY THE LATERAL LINE CANALS AS SEEN EXPERIMENTALLY IN THE GOLDFISH (*CARASSIUS AURATUS*)

GEORGE MILTON SMITH

Anatomical Laboratory, School of Medicine, Yale University

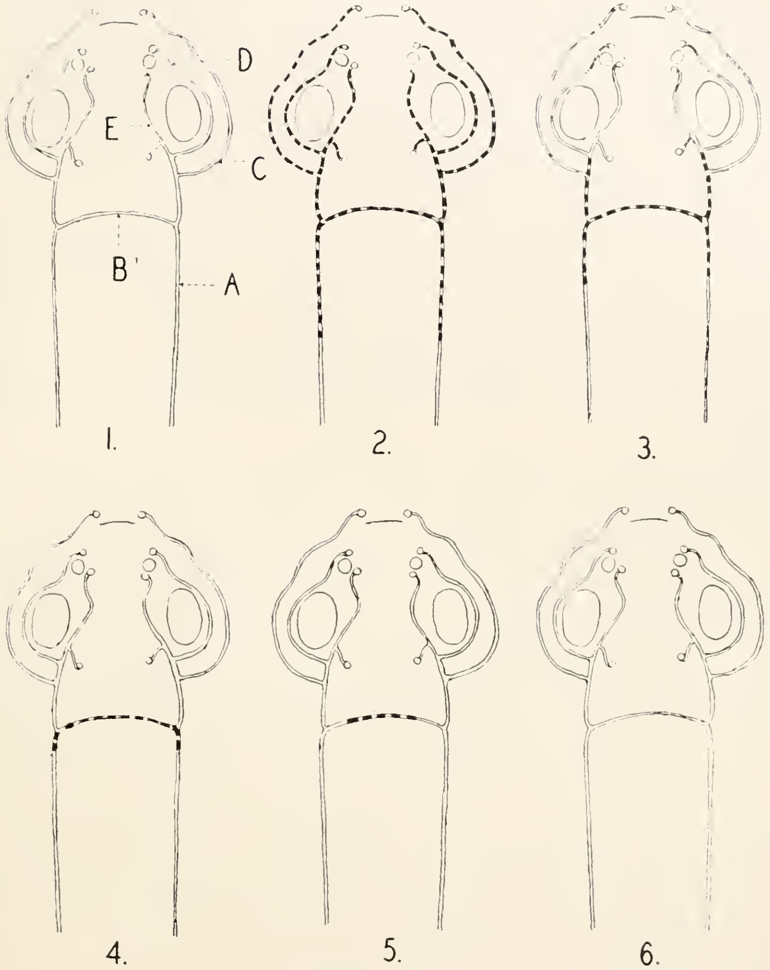
In the course of studies of lateral line canals of the goldfish, it seemed advisable to observe possible reactions of the canals of the lateral line organs to absorption of coloring substances held in suspension by water. To accomplish this purpose, goldfishes were immersed in various weak solutions containing lampblack, India ink, vermilion and Berlin blue, and allowed to live over periods of time varying from a week to two months. From time to time the fishes were examined and it was found that actually small amounts of these coloring substances had been taken up by the lateral line canals of the head or trunk. Such small patches of absorbed pigment occasionally found caught in the lumen of the lateral line canals gave, however, unsatisfactory evidence of any mechanism of absorption or expulsion of fluids into the system of canals. Finally, by using more highly concentrated solutions of some of these same substances, in which the fishes, temporarily, were allowed to swim, a very striking outline of the lateral line canal system filled with coloring substance was obtained; and there was also offered an opportunity of directly observing the intake and expulsion of these colored fluids through the pores distributed along the canal system.

An illustrative experiment is as follows: goldfish, length 5 cm. from tip of snout to base of tail, whitish color. Solution: India ink 20 cc., water 200 cc., temperature 20° C. Preliminary examination of fish showed normal-looking lateral line canals of head and of body. The fish was placed in the India ink solution for thirty seconds, rinsed in water, and changed to a shallow dish of water for examination under the dissecting microscope. The canal system of each side of the head was sharply outlined in black, the supra and infraorbital, the hyomandibular and the supra-temporal canals were deeply injected, and appeared as sharp black lines. The absorbed India ink extended to about one-fourth of the adjacent region of the lateral line of the trunk. After the lapse of over one half minute, there was noted black coloring matter, stringy as if mixed with mucus, first at one and then another of the pores of either side of the head. The canals a few minutes later, began to assume here and there a clearer, grayish appearance. Bits of coloring matter were wiped away with cotton swabs from the pores and were followed by fresh extrusion of delicate shreds of darkly colored mucus. The fish was now allowed to swim in a large jar of clear water at room temperature. At the end of 10 minutes the hyomandibular, supra and infraorbital canals were clear of India ink. At the end of 15 minutes the lateral canals of the trunk had nearly cleared. At the completion of 30 minutes, only the supra-temporal canal showed the remains of India ink in the form of a faint gray line. The supra-temporal canal was cleared of the remaining India ink when 35 minutes had elapsed, so that all canals now contained a clear, limpid, normal-looking mucus with no evidence of previous staining (Figs. 1–6).

The immediate penetration of colored fluids into the canal system may be observed under a dissecting microscope by applying drops of India ink, by means of a finely drawn pipette, over the pores of any part of the canal system of the head or trunk. There follows a rapid intermingling of India ink with the mucous contents of the canals and a consequent spread of India ink along the canals in either direction from the point of application of ink at the pores of the surface. If the application of India ink is continued, adjacent communicating branches of the canals soon become injected with the black coloring substance. When the application of India ink is discontinued, expulsion of the India ink, mixed with mucus, begins and can be seen leaving the canals at the pores which furnish communicating passages between the canals and surface. Elimination of India ink, mingled with mucus, continues until the canals are entirely cleared and appear normal.

It is essential to employ healthy, active goldfishes for experiments of this character. Dying fish take up coloring substances in an irregular manner. It was found that in the dead goldfish a penetration of coloring substances occurred to some extent. This seemed to be less intense and more irregular and patchy than in the living fish and, of course, there was not the immediate elimination of coloring substance by the flow of mucus from the canals. At times no penetration of the coloring substances occurred in the case of the dead fish, possibly on account of the lack of mucus in the canals.

Experiments such as these mentioned above were repeated many times in different ways with evidence of intake and expulsion whenever coloring substances were brought into contact with the lateral line canals. This evidence occurred also in the experimentally-blinded fish and in fishes with nares destroyed by cautery.



FIGS. 1, 2, 3, 4, 5 AND 6. Diagrammatic drawings of lateral line canals of goldfish as seen from above, illustrating intake and expulsion of a solution of India ink, 20 cc.; water, 200 cc. Fig. 1, *A*, Lateral line canal of trunk; *B*, *C*, *D*, and *E*, supra-temporal, hyomandibular, infraorbital and supraorbital canals, respectively, previous to intake. Fig. 2. Filling of canals, after 30 seconds of immersion in India ink solution indicated by black dots in canals. Fig. 3. Clearing of supraorbital, infraorbital, and hyomandibular canals 10 minutes after fish was placed in clear water. Figs. 4 and 5 show progress of clearing after 15 and 30 minutes respectively. Fig. 6 shows canal system entirely cleared after 35 minutes.

India ink and Berlin blue acted as coloring agents most favorable for the experiments. Vermilion in suspension in water was useful for the studies over longer periods of time when certain symmetrical distributions of absorbed coloring matter occurred. Lampblack was not found satisfactory. It rarely gained entrance into the canals, possibly because the conglomerate and adherent particles formed were too large to permit of entrance into the pores of the canal system.

The complete elimination of absorbed coloring substances from the lateral canals varied in different animals over a considerable range of time. Such a difference in the elimination of India ink from the lateral canals was noted in the following experiments, carried on simultaneously with two fishes of different size:

Two goldfishes, *A* and *B*, 4½ cm. and 7 cm. respectively in length; fluid for immersion: India ink, 100 cc.; water, 500 cc.; temperature, 20° C.

1:27 P.M. Both fishes placed in India ink solution.

1:30 P.M. Both removed and examined. In both, all branches of lateral line system of head were colored black. The lateral lines of the trunk were black in the proximal or cephalic third in both fishes.

1:31 P.M. Placed in tanks of fresh water. Both fishes, from now on, examined under the binocular microscope every 10 minutes.

1:41 P.M. In both fishes the lateral canals of the body were cleared of black color, and in both the nasal parts of the supraorbital canals and the submaxillary parts of the hyomandibular canals were clear.

2:11 P.M. Clearing of canals had proceeded to a point where *A* showed only a moderate amount of India ink in the supratemporal canal; and *B* showed a very slight staining of the supratemporal, both infraorbitals and the posterior part of the hyomandibular on the right and left sides.

2:31 P.M. Fish *A* showed only a slight amount of staining in the occipital canal, while fish *B* had all canals perfectly cleared and translucent.

2:51 P.M. Fish *A* had canals now entirely cleared of India ink, having taken twenty minutes longer than fish *B*.

Apparently, with a change in environment, the lateral canals of the goldfish were placed in operation as forms of testing apparatus. If the fish was changed from one colored solution to another of different color, directly, or with an opportunity of cleaning the canals in fresh water, the lateral line canals took up the colored fluid of the new environment.

An experiment illustrating a change involving intake and expulsion of three different colored solutions is the following:

Goldfish, 5½ cm. in length; markings: whitish with slight black pigment above eyes.

6:41 P.M. Placed in a dish containing vermilion, 20 grams; water, 500 cc.

6:54 P.M. Left supraorbital canal was brilliantly injected with vermilion and there was a small amount of vermilion in the right hyomandibular canal near the angle of the jaw.

7:00 P.M. Same distribution of vermilion as at previous reading. Fish changed to clear water.

7:15 P.M. Left supraorbital canal clear of vermilion. Minute plug of vermilion in right submaxillary region.

7:15 P.M. Fish placed in a dish containing Berlin blue, 5 grams; water, 500 cc.

7:18 P.M. After 3 minutes taken out of Berlin blue solution. Both infraorbital canal and submaxillary parts of hyomandibular canal showed as bright blue.

7:19 P.M. Placed in fresh water.

7:29 P.M. Canals cleared of all traces of Berlin blue while in fresh water for 10 minutes.

7:30 P.M. Placed in a solution containing India ink, 100 cc.; water, 500 cc. for one minute.

7:31 P.M. Removed from India ink solution (1 minute). All lateral line canals of head and side were black.

7:31 P.M. Fish placed in clear water.

8:40 P.M. Canals of head and trunk now appeared completely cleared of India ink, the lateral line canals having absorbed and expelled three different colored solutions in the space of two hours.

Similar results were noted in another fish (5 cm. in length; whitish silvery color) which had been placed, six weeks previously, in a solution of Berlin blue, 5 grams; water, 2500 cc. With all canals deeply stained blue this fish was changed directly to a solution of India ink 20 cc.; water, 200 cc. After one minute in India ink the nasal parts of the supraorbital canals and the anterior regions of the hyomandibular canals on both sides were black and readily distinguishable from the adjacent blue. Returned to the same Berlin blue solution in which it had been swimming for six weeks, the India ink in the above-mentioned canals could no longer be recognized at the expiration of 20 minutes; all the canals of the head and trunk were again stained a bright blue.

When goldfishes were kept in an environment of colored fluid for longer periods of time, such as one week to two months, the absorbed coloring matter in the lateral canal system varied from a condition of complete filling of the canals to one where only certain branches were incompletely filled. Occasionally no coloring appeared in any of the canals. In other words, the mucous secretion of the canals may clear away previously absorbed coloring substance and keep the canals partly or completely clear in spite of the fact that the fish is living in a colored solution.

In the following experiment a goldfish was allowed to remain for one month in a solution of Berlin blue. When placed in fresh water at the expiration of that time, clearing of the canal system seemed unusually long (3 hours and 20 minutes.) Tested immediately afterwards for elimination of India ink, this substance was also slowly expelled (3 hours and 8 minutes). The time of intake did not seem to be affected.

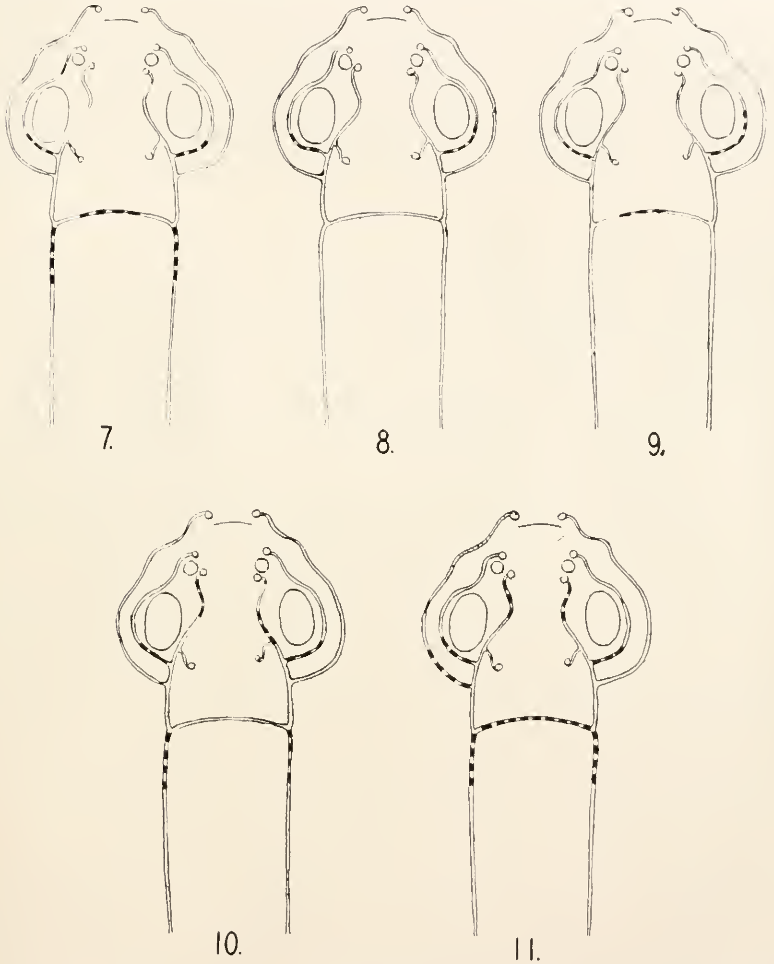
Experiment: 11/30/29. Goldfish, whitish silver in color; 4 cm. in length was placed in a jar containing Berlin blue, 5 grams; water, 2500 cc. The lateral line canals of the head and body were stained a vivid blue in 30 seconds. Examined from time to time during the first three weeks, the fish showed variations in distribution of blue in different branches. Examined daily for the last seven days of a thirty-day period, all lateral line canals of head and trunk were intensely stained with blue.

12/30/29. After a month immersed in Berlin blue solution, with all the canals deeply stained blue, the fish was placed in clear water. In 3 hours and 20 minutes, all the canals of the head and body were clear of blue color. Changing the environment now to one of India ink (20 cc.; water, 200 cc., the canals became quickly and completely stained black in 30 seconds. Returned to clear water, the canals were freed of India ink in 3 hours and 8 minutes. Returned finally to the original Berlin blue solution where the fish had lived previous to the present experiment for a period of one month, the canals took up an intense blue stain in 30 seconds.

In goldfishes kept in a solution containing vermilion, the intake of red-pigmented particles was more leisurely performed, appearing in small patches in the course of the first twenty-four hours. Two fishes which were examined from day to day, during a period of two months, showed various branches irregularly filled with vermilion mixed with mucus contained in the canals. Not infrequently the absorbed vermilion was bilateral in distribution and symmetrically arranged in the different canals of the head and trunk. This symmetrical distribution of vermilion in the lateral canal system of a fish kept in a solution of vermilion, 10 grams; water, 3000 cc. for two months is indicated in the accompanying figures (7–11) based on daily observation for 5 days when a symmetrical pattern of intake happened to be present.

Discussion and Summary

It is not essential for the present purpose to state in detail the historical data of the lateral line canals and organs. It may not be amiss,



Figs. 7, 8, 9, 10 and 11. Diagrammatic representation of canal system of goldfish kept in a solution of vermilion, 10 grams; water, 3000 cc., for a period of two months. Absorbed pigment, although usually irregularly distributed in lateral line canals, appeared symmetrically arranged in this instance during a period of five consecutive days. The canals dotted in black contained absorbed vermilion.

however, to recall that the presence of lateral canals in fishes, as cited by Fuchs (1895) was known and described by at least three anatomists of the seventeenth century,—Nicolas Stenonis (1664), Lorenzini (1678), Rivinus (1687).

The lateral line canals were generally regarded as mucous canals or Schleimkanäle until the time of Leydig (1850–51), whose careful histological studies of the contained end organs led him to the conclusion that the lateral organs were sensory organs. Since that time a vast amount of data has accumulated as the result of the work of many investigators, and reviews on the subject appear in connection with the important works of Ayers (1892), Fuchs (1895), Allis (1904) and Johnson (1917). From the functional standpoint, Lee (1898) has stated that there has been no concensus of opinion as to the exact function or mode of action of the lateral line sensory organs. His own conclusions were that the lateral lines have a sensory function which is closely connected with the motor organs and is analagous to the function of the ear, and hence they may be regarded as organs of equilibrium. Schulze (1870) had suggested earlier that this sense perception was possibly an appreciation of mass movement of the water or of movement of the body through the water; whereas Fuchs (1895), from carefully conducted researches, was led to the conclusion that the lateral line sensory organs gave sensory impressions of changes in hydrostatic pressure. Hofer (1908) believes from his studies that the lateral line organs are stimulated alone by weak currents of water. Parker (1918), in the course of researches conducted on the auditory apparatus, finds that the lateral lines respond to water vibrations which are slower than those which affect the auditory mechanism.

Recent views of the lateral line sense organs place their function, according to Herrick (1927) intermediate between tactile and auditory organs. Their nerve supply, he states, is from the lateralis roots of the seventh and tenth cranial nerves. He points out the intimate association with the eighth nerve supplying the internal ear, and the termination of these nerves in the acoustico-lateral area of the medulla. According to Herrick (1927), the structure of the end organs of the lateral line system and those of the human ear are of the same type.

From the experiments carried out on the goldfish cited in the present communication, it would seem that there is in the lateral line canals of the goldfish, demonstrable by the experimental use of colored fluid, a mechanism of intake and expulsion of fluids. The intake is rapid and seems to vary from a few seconds to a few minutes. The elimination from the canals is slower and more deliberate, taking from fifteen minutes to one hour or more. Colored fluids in passing through the pores of the lateral canals mix rapidly with mucus existing in the canals, the mucus acting possibly as a diluent. The discharge of coloring substance from the canals is effected by an outward discharge of mucus through the pores of the canals. The mixture of colored material and mucus appears in the form of delicate colored shreds or plugs as they are expelled. These colored mucous shreds quickly wash away in surrounding water.

Therefore, experiments, such as these described, where lateral line canals take up and expel different coloring substances in suspension when the fish is changed to solutions of different color, suggest that the lateral canals of the goldfish function, in part, at least, as sensory testing mechanisms for chemical or physical changes in environment; and that the ready flow of mucus from the canals furnishes an efficient means of eliminating fluids that have been tested by the end organs of the canal system.

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RAT VAS DEFERENS CYTOLOGY AS A TESTIS HORMONE INDICATOR AND THE PREVENTION OF CASTRATION CHANGES BY TESTIS EXTRACT INJECTIONS[1]

SUP VATNA

Hull Zoölogical Laboratory, The University of Chicago

I. Introduction

The cytological and histological changes in the prostate glands and the seminal vesicles of the rat following castration have been worked out by Moore, Price and Gallagher (1930) and Moore, Hughes and Gallagher (1930) respectively, and it was found that there are some dependable criteria, by which one can tell whether the sex hormone is present or absent. It is desirable to know what other organs may be affected and if the changes will be consistent enough to serve as a sex hormone indicator. This paper will deal with the study of the vas deferens of the white rat in its normal state and after different periods of castration, and the effects of subcutaneous injections of extracts from the testicle upon the castrate condition.

This study was suggested to me by Prof. Carl R. Moore as another unit in the program of sex studies now being carried on in the Departments of Zoölogy and of Physiological Chemistry and Pharmacology. I am grateful to him for advice and assistance given to me throughout the course of the work. I will show in this paper that the structure of the vas deferens is controlled by the internal secretion of the testes and furthermore that this control can be maintained in the castrated animals by means of subcutaneous injections of the extracts of bull testes. A preliminary account of the findings has already appeared (Moore, Vatna and Gallagher, 1930). The numbered preparations of bull testis extract were supplied in strengths unknown to us until after assay. They were prepared by Mr. T. F. Gallagher under the direction of Professor F. C. Koch in the Department of Physiological Chemistry and Pharmacology, to both of whom is expressed a debt of gratitude. The earlier papers from these laboratories (McGee; McGee, Juhn and Domm; Moore and McGee; Moore and Gallagher; Moore, Price, Hughes, Gallagher; Gallagher and Koch; Moore, Gallagher and Koch) have presented the biological test methods previously employed, and the methods of hormone extraction, and the reader is referred to them for details.

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[1] This investigation has been aided by a grant from the committee on research in problems of sex of the National Research Council; grant administered by Prof. F. R. Lillie.

Other laboratories have recently reported positive results from attempted hormone extraction from the testis of various mammals and the urine of men (Martins and Rocha e Silva, 1929; Loewe and Voss, 1929; Funk, Harrow and Lejwa, 1929, 1930).

II. Material and Method

White rats were used in this experiment. The study involves the examination of the vas deferens from about thirty normal animals of varying ages, thirty-five castrated, and fifty castrated injected animals.

Castration was performed through a mid-ventral abdominal incision. In some cases the body of the epididymis was cut through, leaving the tail of the epididymis attached to the vas deferens. With others the entire epididymis was removed with the testis.

The proximal, or urethral end of the vas deferens presents a structure that shows more marked effects from castration than does the distal, or epididymal end, hence the proximal two-thirds of this reproductive tube has usually been the part that has received the greatest attention.

The tissues were fixed for histological study in Bouin's fluid and Zenker formol mixture. Bouin's fluid was found to be the better of the two, and therefore was used throughout the work. The sections were cut at 4μ thickness and were stained in such mixtures as Delafield's hæmatoxylin with eosin as a counter stain, or iron hæmatoxylin, or Mallory's triple stain.

Mann's osmo-sublimate fixative was also used to demonstrate the Golgi apparatus. The technique employed was that of Ludford's (1925, 1926) modification of the Mann–Kopsch method. Briefly, the vas was cut into small pieces of about three mm. in length and fixed in a freshly prepared mixture of an equal volume of one per cent osmic acid in distilled water and a saturated solution of mercuric chloride in normal salt solution, for about twenty hours. The tissue was then washed in two changes of distilled water for about thirty minutes, and placed in two per cent osmic acid solution in quantities sufficient to cover it, after which it was placed in the dark at room temperature for about seven days. At this time the osmic acid solution was discarded, the tissue washed once in distilled water, and transferred in distilled water, to an oven at about 35° C. for four days. The tissue was next washed in running tap water over night, and then put through the ordinary histological procedures, such as dehydration, clearing, imbedding, and sectioning. The sections were bleached in a solution of hydrogen peroxide in 95 per cent alcohol.

III. The Structure of the Normal Vas Deferens

The vas deferens of the rat is more or less spindle-shaped in external form. Between the urethral end and the middle of the vas, is an elongated swollen region, from the distal end of which the tube tapers toward the epididymis and from the proximal end toward the urethra.

In the normal, the vas is always full of spermatozoa. This can be detected with the naked eye because of the milky white streak which is present in the middle throughout its length. The swollen region is especially distended by spermatozoa.

The normal vas deferens has been studied both from animals sacrificed for the purpose and from animals after unilateral castration of varying periods. The latter type has been used in order to see whether the spermatozoan content in any way modified the structure of the epithelial lining. In the mammals there is no question now as to the ability of one testis to keep up the normal state of the accessory reproductive organs. The vas deferens from the latter group is preferred for the sake of comparison, although there is no essential difference between the normal histology of the vas from the two sources mentioned, except when the spermatozoa have collected in an unusually large quantity. Then the height of the epithelium may be slightly lowered due to the distention of the lumen in general, but the arrangement of the nuclei of the epithelium is not at all disturbed. The cilia may be somewhat distorted from normal shape. However, in all cases examined, their appearance is decidedly not that of a castrate type.

The vas deferens of most mammals, as generally known, is not ciliated; some species, however, are well furnished with cilia. The mouse and the rat belong to the latter group. The word " cilia " in connection with the vas deferens, Benoit (1926) thought should be " stéréocils " or " poils," on account of their non-vibratile nature. The short term " cilia " will be used in this paper to mean " cilia-like " structures.

The histology of the vas deferens is a very simple one. The tube consists of three easily distinguishable layers, the outside muscular layer, the mucous, and the epithelial or inner layer. The outer coat covered by peritoneum consists of longitudinal and circular muscle layers, and makes up approximately four-fifths of the thickness of the walls of the tube. Internal to the muscular layer is the so-called mucous layer composed primarily of connective tissue-like cells and blood vessels. This layer is sensitive to operative manipulation which is in no way related to hormone control. From an unoperated animal, it is narrow and the cells are more or less tightly packed together, whereas the vas from a unilaterally castrated animal has a much broader mucous layer and the cells are rather scattered. The internal epithelial layer bordering the small lumen is definitely separated from the mucous layer by a very thin cord of about one or two cells in thickness. This cord of cells forms the outline of the basal part of the epithelium, and will be referred to in this paper as the " basement-cell layer."

The epithelial layer is composed of tall columnar cells, resting upon a distinct basement-cell layer, and the free end of the cell is covered by a heavy mass of cilia-like structures projecting into the lumen. The nuclei of the cells are generally oval in shape and variable in chromatin constituents. They vary slightly in position in the vasa of different animals, but in any one animal they occupy the same relative position in all of the cells. Thus the nuclei are seen to form a definite layer paralleling the basement-cell layer (see Figs. 1 and 5). At many places in a section one observes a few nuclei that seem to be differentiating from the basement cells, with others present above the nuclear layer apparently migrating toward the lumen. In the lumen itself, one often finds a group of epithelial cells in various stages of degeneration. These findings suggest a series of changes in the normal vas deferens, wherein cells are added to the epithelium from the basement-cell layer, and at the same time others having functioned actively for a certain time, are thrown off into the lumen, where degeneration occurs.

Between the nucleus and the ciliated border of each cell, the cytoplasm is of a condensed homogeneous granular character, whereas that basal to the nucleus is much less dense and is fibrillar in character. The difference between the distal and proximal ends of the epithelial cell is very marked. The finely granulated material in the distal portion is believed to be made up of secretory products (Myers-Ward, 1897; Benoit, 1920). Benoit (1926) by the use of a special technique found certain definite lipoid bodies which he called " parasomes " in the epithelial cells of the vas deferens of the mouse and rat. These " parasomes " were believed to be the product of protoplasmic differentiation. They first appear when the animals are about fifteen days old, and in the adult they are found scattered throughout the cell. He suggests that the " parasomes " normally undergo some sort of dissolution and contribute to the formation of a liquid product of secretion. The investigation reported here has not involved a study of these " parasomes."

There is always a small amount of secretion present in the lumen of the vas in normal animals. This secretion forms a finely granular homogeneous mass and stains with eosin.

The vas deferens prepared by the Mann-Kopsch technique reveals definite, well-formed Golgi bodies in the epithelial cells. The Golgi bodies are located approximately midway between the nucleus and the lumen end of the cell and are of the reticular type. Their characteristic shape is shown most clearly in slightly under-impregnated sections, in which case the threads making up the reticulum will be blackened only on the outside, thus giving a double-lined appearance. The size of the Golgi bodies in the normal is about that of the nucleus, though they may be somewhat larger in some cases.

IV. Changes in the Vas Deferens Following Castration

In order to determine whether the vas deferens was affected by castration, I have studied preparations from animals in a closely graded series from three days up to seven months after testis removal.

The tissue prepared from animals sacrificed at 3, 5, 6, 7, and 9 days after castration is essentially normal. The gross size, relative thickness of the layers, the character of the epithelium and the condition of the cilia do not differ markedly from the normals.

The Golgi bodies, however, begin to show some differences for they become smaller in comparison to the size of the nuclei, and, more striking, the reticulum breaks up to form a group of crooked rods or coarse granules.

At 10 and 15 days after castration, the gross size as well as the histological structure of the vas of some animals shows a decided change, characteristic of a longer time castrate. The vas deferens becomes smaller and the epithelium may be typical of a 20-day castrate. However, other animals castrated for this period may retain essentially the normal condition in the vas.

The Golgi bodies after ten to fifteen days of castration have undergone a marked fragmentation. The portion of the cells where the Golgi bodies are normally found will be seen to be full of scattered osmiophilic granules. These granules may clump together, but the structure does not suggest a normal Golgi apparatus.

Twenty days after testis removal the vas deferens characteristically shows the effects of castration. This period is of special importance inasmuch as many of the effects of testis extract injection have been studied for this period of time after operation.

The size of the vas is now noticeably smaller, due to the degeneration of the muscular layer, which normally makes up almost the whole thickness of the tube. The morphological structure of the mucous layer has no constant bearing upon castration.

The most apparent changes occur in the epithelial layer. The absolute height of the epithelium from the basement-cell layer to the luminal border is slightly reduced. The cell walls are no longer clearly visible, and the nuclei instead of forming a well-defined layer paralleling the basement-cell layer are now more closely aggregated in an irregular distribution giving the appearance of pseudostratification. The epithelium now appears as a syncytium.

The nuclei show little, if any, reduction in size, but because of the reduction in the amount of cytoplasm in the cells, they now lie close to the basement-cell layer. The cytoplasm between the nucleus and the lumen end of the cell is likewise greatly reduced.

The ciliary border of the epithelium also differs greatly from the normal. The cilia are in most cases completely absent from the vas deferens of 20-day castrate animals (see Fig. 6). In a few others they may still be present but greatly reduced both in number and length and present often an interwoven, irregularly twisted condition.

The secretion found in the lumen does not seem to be changed in quality, but is much reduced in quantity following castration. However, even after long-time castration, there is always a small amount of secretion present. Benoit (1926) reports from his study on mice and rats that the parasomes, the bodies responsible for the formation of secretory products, disappear completely after thirty days of castration. From our own study on the rat, we have been unable to confirm the statement regarding the absolute cessation of secretion.

The Golgi bodies too are decidedly different from the normal at this period of castration. Their gross size, relative to the size of the nucleus, is very much reduced. The former reticular arrangement has usually changed to a granular one, and these granules sometimes form an irregular cap over the end of the nucleus.

The typical condition of the twenty-day castrate animal given above holds for the majority of animals castrated for this period, but occasionally slightly different conditions may be encountered. A few apparently more resistant animals have suffered less from castration than others and appear almost normal, except for a lower epithelium and a slight crowding and displacement of the nuclei.

The typical degenerate condition of the vas deferens at twenty days after testis removal represents, with some exceptions, essentially the condition that is to be found in later castrates. The series which I have studied includes animals castrated for periods of 21, 25, 30, 33, 40, 50, 60, 80, 110, 150, and 210 days. As the age of castration increases there is little, if any, increase in the amount of involution.

Fig. 7 shows the condition of the vas deferens in an animal castrated for two hundred and ten days and in comparison with the normal (see

Fig. 5), clearly shows the absence of a ciliary border of the epithelium, the lowered height of this layer, the apparent stratification of the nuclei, the involuted mucous layer and the reduced muscular layer. Fig. 2 in comparison with Fig. 1 demonstrates clearly the difference between a five-month castrated vas and the normal.

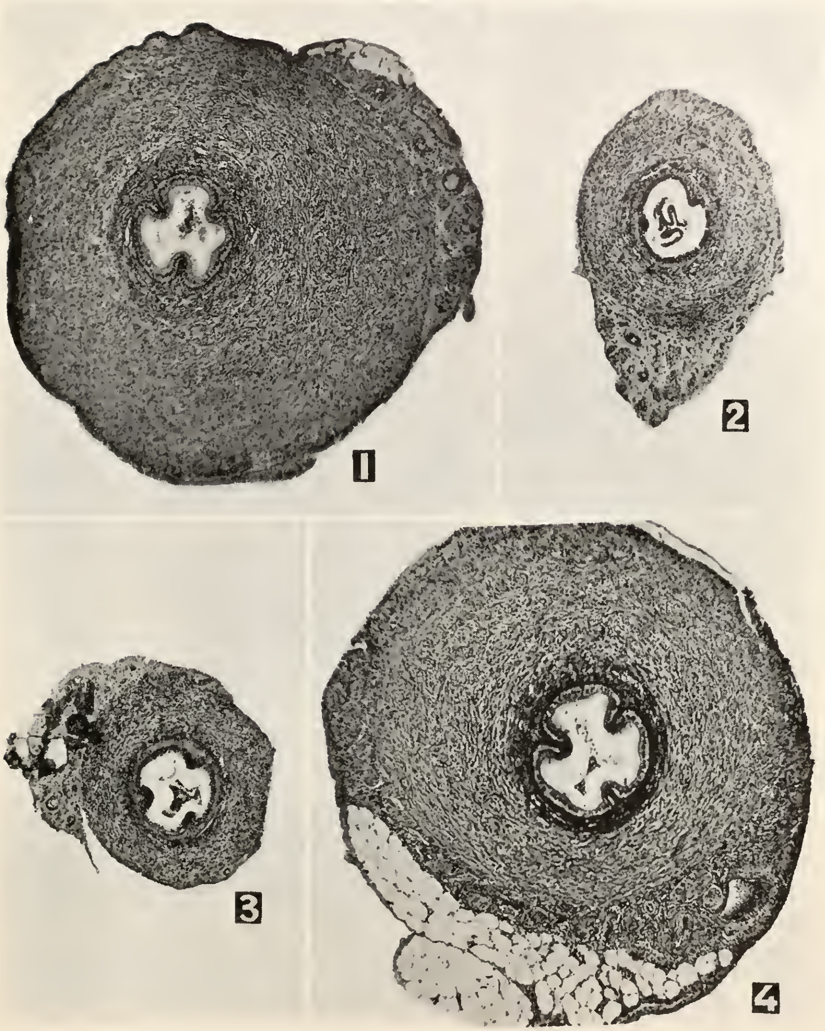
It is apparent, therefore, that castration leads to a marked degeneration of the vas deferens. Since this influence is to be attributed to the endocrine influence of the testis rather than to the gametogenetic influence, we have in this degeneration a means of testing the effectiveness of preparations of testicular extracts. If testis removal is followed by the injection of the testis extracts and the vas deferens remains in a normal condition, it will be apparent that the extracts exercise an influence similar to that of the internal secretion of the testis. My observations on this point are described in the following section.

V. The Effects of Testis Extract Injections

In the preceding section, definite changes have been described for the various parts of the vas deferens. These are: Decrease in gross size, involution of the muscular layer, slight lowering of the epithelium, the syncytial character of the cells, pseudostratified appearance of the nuclei, loss of the cilia, and reduction in size of the Golgi bodies, with accompanying fragmentation.

Early work from these laboratories supplies proof that the active principle of the internal secretion of the testes is contained in suitably prepared lipoid extracts of the glands of the bull. In the course of this study, many samples of the extracts have been used for injection on over fifty castrated males. Some of these were less potent than others, depending on the preparation methods and the dilution of the samples. The results, therefore, are of a wide range. The typical positive cases to be described were chosen from animals having received appropriate strength of the hormone solutions.

Since twenty days was found to be the period at which the degenerative changes of the epithelium reach their height, it was selected as minimal length of time for testing the hormone extracts. Animals have been injected daily immediately after castration in order to see whether the effects of testis removal could be indefinitely postponed. In addition to this procedure, other animals have been castrated and permitted to develop the castration condition with subsequent injection to test the capability of the extracts to restore the degenerate to a normal condition. This latter procedure has been followed in the case of animals castrated as adults as well as those castrated before puberty.



Cross-sections of rat vas deferens. Photomicrographs of Bouin-hæmatoxylin preparations. About 50 × before reduction. (All photomicrographs were made by Mr. Kenji Toda.)

1. From a normal animal.
2. From a five month castrate.
3. From a 110-day prepubertal castrate.
4. From a 110-day prepubertal castrate, given forty daily injections of bull testis extract.

1. *The Maintenance Experiment*

In this series, the animals were given twenty daily injections, or more in some cases, immediately after castration to maintain the normal condition.

The histological study of such injected castrates shows a normal structure of the vas. The epithelium is simple columnar and abundantly supplied with cilia, and the nuclei have the simple regular arrangement, typical of the normal. The Golgi bodies are approximately normal.

2. *The Repair Experiment*

a. *Prepubertal castrates*

Two series of prepubertally castrated animals have been utilised for injection. The first group of four animals was castrated at fourteen days after birth and the second group of five animals was castrated at forty days of age. The second one is more instructive, hence it will be described in detail as to the procedures. Five animals of the same litter were castrated at forty days after birth, and at one hundred days after castration, four animals were injected with the testis extracts No. 8922,—one-half cc. being injected daily. When the injections had been given for ten days, one of the four injected animals was killed, and at the same time the uninjected control was also killed. At twenty days after the injections, one of the three was killed. The next one was killed after having received thirty daily injections, while the last one was killed at forty days.

The results of the study of the experimental series are as follows:

The uninjected control showed every sign of a castrated condition (see Fig. 3), with the typical loss in gross size, changes in nuclear arrangement, lowering of the epithelium, etc.

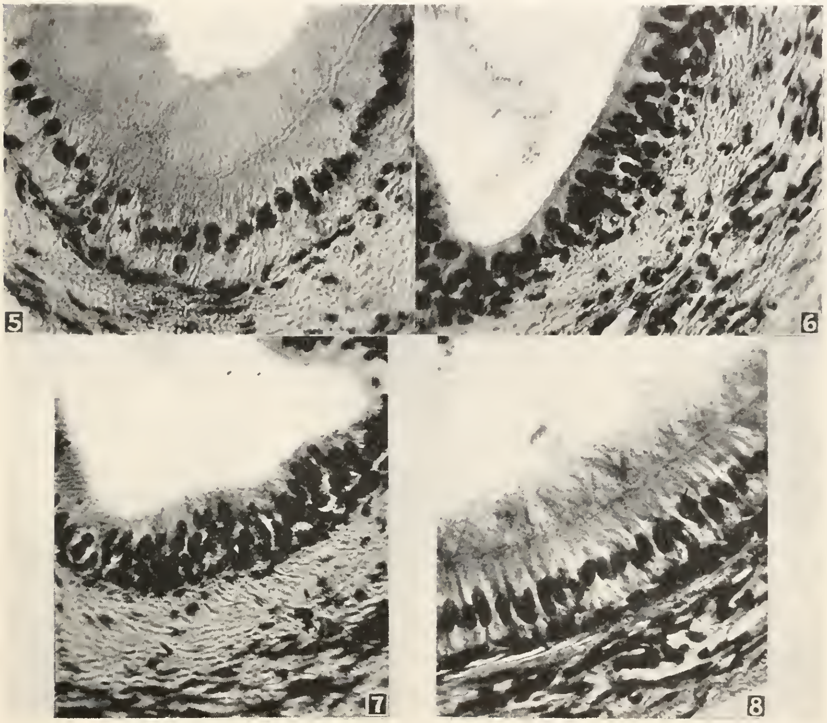
The vas deferens of the 10-day injected animal resembles the castrate type except that it shows an increase in the height of the epithelium with a partial disappearance of the pseudostratified effect. The secretion in the lumen and in the distal ends of the epithelial cells is greater in amount.

In the 20-day injected animal the vas is nearer normal in that it shows a strikingly high epithelium, with a fair amount of cilia.

The 30-day vas is indistinguishable from that of a normal, as far as the structure of the epithelium is concerned. The size of the vas as a whole is considerably larger than its castrate control but not as large as the normal.

The vas deferens from the 40-day injected prepubertal castrate is normal both in structure and size. The diameter of the entire vas is now double that of the control (Fig. 4).

This study shows that the prepubertal castrated vas deferens responds definitely to the introduced testis extract as do the adult castrates and returns to the normal condition in forty days despite its undeveloped state for a period of about one hundred and ten days.



Cross-sections of rat vas deferens. Photomicrographs of Bouin-hæmatoxylin preparations. About 650 × before reduction.

5. Portion of Fig. 1. (Normal animal.)
6. From a 20-day castrate.
7. From animal No. 96—tissue removed seven months after castration.
8. From same animal (No. 96), which had received thirty daily injections of bull testis extract after the removal of the tissue shown in Fig. 7.

b. Adult Castrates

A number of adult animals were castrated and allowed to remain for various periods of time before injections were begun. The introduction of testis extracts has always served to return the vas deferens to the normal condition provided the concentration of the lipoid extract was sufficiently great.

The results of injecting the extract into long time castrates will be illustrated by reference to one animal (No. 96). This animal was castrated and seven months later was operated upon for removal of one vas deferens to serve as the control, and its condition is shown in Fig. 7. The animal was then subjected to testis extract injection daily for a period of thirty days; one-half cc. was injected subcutaneously each day. It was killed and the opposite vas deferens removed to show the effects of the injection. A cross section of the vas after injection is shown in Fig. 8, and should be compared with its mate removed before injections were begun (in Fig. 7). It can be seen clearly that whereas the seven month castrated vas deferens is in a highly degenerate state, its partner has been returned to the normal condition by means of the injections. A second animal treated similarly, but injected for a period of only twenty days, showed that the vas deferens had returned to an almost normal condition within this period. When castration has been of shorter duration, injections have been followed by similar return to the normal condition.

VI. Discussion

In this study we have demonstrated that the vas deferens is also under the control of the sex hormone for its normal maintenance, as was shown to be the case for the prostates and the seminal vesicles by Moore, Price and Gallagher (1930) and Moore, Hughes and Gallagher (1930) respectively. If the hormone-producing glands—the testes—are removed, certain definite degenerative changes set in, and these changes are maximal by about twenty days after testis removal.

The vas reacts more slowly to castration than do the seminal vesicles and prostates of the rats and therefore has not provided as delicate a method for hormone assay, nor one as easily read as the light area of the prostates or the secretion granules of the seminal vesicles. Although the changes following castration do not appear as rapidly in the vas, they are as definite as those that appear in the other accessory reproductive glands that have been studied. The vas responds positively to potent injections of testis extract, therefore it provides a supplementary test for the presence of the male hormone.

In other sections of this paper, data have been presented showing that by injections (1) vasa of castrated animals have been maintained at the normal level, (2) vasa that had been allowed to regress for seven months after castration have been built up to normal, and (3) vasa of prepubertally castrated animals have been allowed to regress for one hundred and ten days and have been built up to a normal functioning state in forty days.

One experiment was described in detail in which a rat was castrated and after seven months one vas was removed and the other remained to be removed after thirty days of injections. The former was a typical castrate, and the latter showed a condition normal in every respect. From these data, there can be no doubt that the active principle of the testis has been supplied by testis extract injections.

With varying potencies of hormone, the results of injections varied from negative effects to complete replacements of the vas to the normal state. The epithelium itself is more sensitive and responds more readily to hormone injection than does the muscular layer and consequently the vas may return to an approximately normal condition while the gross size is below that of the normal. This same condition obtained in the prostate and the seminal vesicles.

Since, by testis extract injection, the vas can be maintained in a normal state as is proved by histological and cytological study, it provides us with another male hormone indicator method to add to those already developed—the spermatozoön motility test, the electric ejaculation test, the seminal vesicle test, the prostate cytology test, and the capon comb growth test.

VII. Summary and Conclusions

1. The vas deferens can be used as a male hormone indicator because it is under the control of the internal secretion of the testis.

2. After castration, definite regressive changes take place within twenty days in all animals.

3. These changes involve:

a. Reduction in gross size through regression of the muscular layer of the vas.

b. Diminution of the amount of secretion in the lumen.

c. Reduction in epithelial height.

d. Loss of the cilia covering the epithelium.

e. Crowding together of the cells and obliteration of the cell walls.

f. Stratification of the nuclei.

g. Great reduction in the amount of cytoplasm in the cells.

h. Changes in the Golgi bodies involving loss in gross size and fragmentation of the Golgi material into rods or granules instead of the typical reticulum of the normal.

4. All these changes can be prevented from developing in the castrated animal by daily injections of suitably potent male hormone prepared from the lipoid fraction of fresh bull testes and dissolved in olive oil.

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5. If the changes have been allowed to develop, the vas can be built up to normal by daily injections of testis extracts.

6. In animals castrated before puberty and allowed to regress for one hundred and ten days the vas can be built up to a normal functioning state by injections; a process which involves bringing the undifferentiated duct to a normal adult state.

7. Injections of pure olive oil fail to prevent castration changes, therefore the potent factor lies in the hormone itself.

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ON *DISTOMUM VIBEX* LINTON, WITH SPECIAL REFERENCE TO ITS SYSTEMATIC POSITION

H. W. STUNKARD AND R. F. NIGRELLI

Biological Laboratory, University College, New York University

Distomum vibex was described by Linton (1900, 1901, 1905), from the pharynx and intestine of the smooth puffer, *Spheroides maculatus*. For many years this species has been studied as the representative of digenetic trematodes by the classes in Invertebrate Zoölogy at the Marine Biological Laboratory of Woods Hole. Since the early and brief reports of Linton, little or no research has been done on the parasite. The purpose of this study is, therefore, to supplement the earlier descriptions of its morphology and to allocate the species in the system of classification of the digenetic trematodes.

Lintonium New Genus

Distomum Diesing 1850 is the equivalent of *Distoma* Retzius 1782, a name proposed as a substitute for *Fasciola* Linnaeus 1758—and consequently a synonym. Looss (1899) showed that *Distomum* is not a generic but a group name, and with the subdivision and disappearance of the previously accepted genus *Distomum*, the proper generic name and systematic position of *D. vibex* has remained an open question. Since *Distomum* is not a valid generic name, and since the species can not be assigned to any existing genus, we propose the new genus *Lintonium* to contain it.

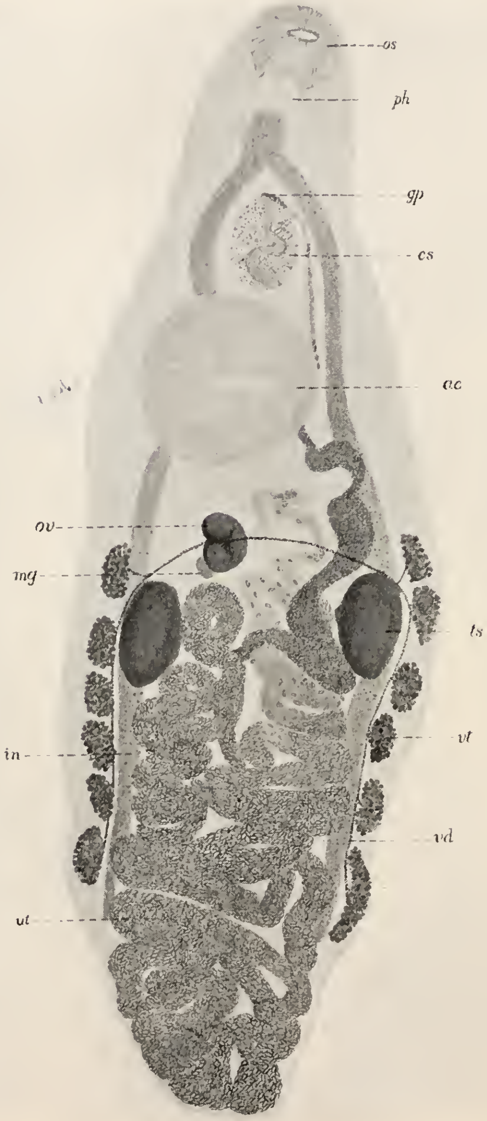
The distribution of *Lintonium vibex*, so far as has been determined, appears to be limited to the species *Spheroides maculatus*, commonly found off the coasts of New Jersey and New York and as far north as Maine. Primarily, however, the members of the group of " swellfishes " are inhabitants of warmer waters, and the relatives of *Lintonium vibex* are presumably to be found, if at all, in species of *Spheroides* which inhabit warmer seas. According to Linton, the largest worms are found in the pharynx, attached to the walls around the entrance to the pouch. Young specimens, however, were encountered in the intestine.

Except for certain details, which appear in the text, our observations agree with those of Linton. The parasites are so variable in size and form that precise measurements are difficult to make. Fixed and stained sexually mature specimens vary from 2 to 7 mm. in length, 0.7 to 2 mm. in width, and 0.266 to 0.912 mm. in thickness. In living worms, the region anterior to the acetabulum is very mobile and may be elongated into a neck-like structure, one and one-half times the length of the body posterior to the ventral sucker. In fixed specimens the acetabulum is located at the posterior end of the anterior third of the body. It is considerably larger than the oral sucker, oval to spherical in shape, and measures from 0.4 to 1.3 mm. in diameter. The suckers are powerful adhesive organs and the parasites are removed from their attachments only with difficulty.

The body wall is strongly developed and the specimens are very muscular. The cuticular covering measures from 0.021 to 0.032 mm. in thickness and is much heavier on the dorsal than on the ventral surface. When the worm is contracted the cuticula is thrown into convolutions that give it a " ringed " appearance, although it is not provided with either scales or spines. The muscular layers of the body wall consist of an external circular, an intermediate longitudinal, and an internal oblique layer of fibers. In the anterior part of the body especially, the parenchyma is traversed by well-developed fibers. These are not arranged in definite layers and have branched or diffuse origins and attachments. Immediately below the muscular wall there are many glandular cells which probably secrete the cuticula. Inside the nuclear zone, on the ventral side of the body, there is a well-developed series of longitudinal muscles that extend from the body wall in the region behind the genital pore to the region of the acetabulum, and others that extend on the region of the oötype.

The mouth opening is subterminal and the oral sucker, spherical to oval in shape, measures from 0.23 to 0.57 mm. in diameter. The pharynx, situated immediately behind the oral sucker, measures from 0.10 to 0.19 mm. in diameter. Following the pharynx there is an apparent esophagus of varying width and diameter. Histologically, however, this structure resembles the digestive ceca; it is lined with epithelium and should properly be regarded as a portion of the intestine. Two simple intestinal crura pass posteriad in the dorsal and lateral regions of the body, terminating blindly about the middle of the posterior third of the worm.

The excretory pore is situated at the posterior tip of the body. It opens from a small vesicle which is lined with cuticula. From the vesicle two collecting tubes pass forward, dorsal and median to the intestinal ceca to the level of the acetabulum where they cross to the extracecal region and continue to the level of the pharynx. The collecting vessels are variable in shape and size and the walls consist of


os
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Lintonium vibex, ventral view × 40. *ac,* acetabulum; *cs,* cirrus sac; *gp.* genital pore; *in,* intestine; *mg.* Mehlis' gland; *os,* oral sucker; *ov,* ovary; *ph,* pharynx; *ts,* testis, *ut,* uterus; *vd,* vitelline duct; *vt,* vitellaria.

a basement membrane bearing a layer of flattened epithelial cells. Further details of the system have not been worked out.

The testes are lateral, situated just behind the middle of the body. Oval in shape, with their longest axis directed anteroposteriorly, they measure from 0.15 by 0.22 mm. in small worms to 0.6 by 0.8 mm. in the largest ones. From the anterior tip of each a vas deferens passes forward on the dorsal side of the body and empties into the seminal vesicle located in the caudal end of the cirrus sac. The cirrus sac is situated on the dorsal side of the body in the region between the bifurcation of the alimentary tract and the anterior border of the acetabulum. The sac has a well-developed fibromuscular wall, containing both circular and longitudinal muscle fibers, and measures about 0.35 mm. in length by 0.22 mm. in width. The seminal vesicle is somewhat coiled, and in some whole mounts gives the appearance of being composed of two parts: a small, oval, caudal portion and a much larger anterior portion. From the vesicle a narrow duct, 0.06 to 0.07 mm. in diameter, leads to the common genital pore. This duct is usually S-shaped and is lined with columnar epithelium. Both vesicle and duct are surrounded by prostate cells.

It is interesting to note that in one instance, a worm was found with a single testis and vas deferens. Otherwise the specimen appeared to be perfectly normal.

The ovary is trilobed; it consists of one large dorsal and two smaller ventral lobes. It is situated on the dorsal side of the body, at the right of the median plane, in front of the testes, and behind the acetabulum. It is slightly longer than broad, measuring from 0.15 to 0.54 mm. in length and from 0.15 to 0.43 mm. in width. The oviduct arises at the posterior tip of the dorsal lobe and just after entering the oötype, gives off Laurer's canal. Laurer's canal passes forward in a winding course and opens to the dorsal surface above the anterior margin of the ovary. It traverses a distance of approximately 0.2 mm., measures about 0.015 mm. in diameter, and is lined with cuticula. After the origin of Laurer's canal, the female duct passes posteriad and ventrad where it receives a common vitelline duct and then turns dorsad and anteriad, to open into the uterus. There is no seminal receptacle. The oötype is enclosed in the cells of Mehlis' gland, which lies posterior and ventral to the ovary. From the oötype the uterus extends laterally and forward. This portion is filled with sperm and light-colored eggs with deeply staining contents. The vitellaria consist of six lobes on each side of the body. They lie in the extracecal area, from the level of the ovary to the caudal ends of the intestinal ceca. Collecting ducts pass forward along the medial face of the five caudal lobes and bend mediad in front of the testes. The cephalic lobes have their own ducts, which discharge into the main longitudinal ducts as they turn mediad. These ducts meet in the median line to form a common vitelline duct that passes through Mehlis' gland to empty into the oötype. No vitelline receptacle was observed. The uterus passes backward on the left side of the body to the caudal end and then forward, and fills the intercecal area behind the ovary with masses of complicated coils. In front of the ovary the uterus continues in the dorsal portion of the body to the genital pore, situated immediately behind the bifurcation of the alimentary tract. The metraterm is short, and there is a small genital sinus into which the male and female ducts open.

The uterus is filled with enormous numbers of eggs. They are ovate in shape, with an operculum at the narrow end of the shell. They measure from 0.045 to 0.054 mm. in length by 0.023 to 0.027 mm. in width.

From the above description the genus *Lintonium* may be characterized as follows: small to medium sized distomes; suckers powerful, acetabulum larger than the oral sucker; strongly muscular bodies, preacetabular region especially mobile; esophagus short or absent, pseudo-esophagus short, lined with digestive epithelium; intestinal ceca extend posterior to the testes; excretory vesicle almost V-shaped with short stem, lateral crura extend to the region of the pharynx; genital pore ventral, immediately behind the bifurcation of the alimentary tract; cirrus sac oval, preacetabular, enclosing seminal vesicle and cirrus; testes lateral, postovarian; ovary postacetabular, lateral and pretesticular; uterine coils extend to posterior end of body, filling the intercecal area behind the oötype; eggs ovate, operculum at the smaller end; vitellaria lateral, postovarian.

In morphological features *Lintonium* agrees more closely with *Steringotrema* Odhner 1911 than with any other known genus.[1] The genus *Steringotrema* was proposed to contain a species described by Nicoll (1909) as *Steringophorus cluthensis,* since the form could not properly be retained in the genus *Steringophorus* because of differences, especially in the form of the excretory vesicle. *Lintonium* differs from *Steringotrema* in several distinct morphological features. The acetabulum, ovary, and testes are much farther forward, and there are differences in the form and location of the vitellaria.

Odhner (1911) proposed a new family, Steringophoridæ, with two subfamilies, Steringophorinæ and Haplocladinæ. In the former he included *Steringophorus* Odhner 1905, *Fellodistomum* Stafford 1904, and the two new genera, *Rhodotrema* and *Steringotrema.* It should be noted, however, that Nicoll (1909) had erected the subfamily Fellodistominæ to include *Fellodistomum* and *Steringophorus*. Consequently, since the two groups are co-extensive, the proposal of the subfamily Steringophorinæ was a deliberate renaming of a previously validly named subfamily. Odhner's reasons for changing the name are stated as follows: "Wenn ich für diese Unterfamilie den von Nicoll (1909, S. 472) vorgeschlagenen Namen Fellodistominae verwenden würde, müsste ich die ganze Familie Fellodistomidae nennen, was mir bei dem Umstande, dass nur ein einziger Vertreter derselben mit der Galle etwas zu tun hat, allzu sinnlos erscheint. In *Steringophorus* erblicke ich weiter diejenige Gattung, welche den Typus der ganzen Familie am reinsten verkörpert; wahrend die typische Art der Gattung *Fellodistomum, F. fellis,* entschieden als der am wenigsten typische Vertreter der ganzen Unterfamilie bezeichnet werden darf. Aus diesen Gründen erscheint es mir als richtig, den Namen Fellodistominae beiseite zu schieben, und ich trage hierbei um so weniger Bedenken da sich dieser Name als erst jüngst geschaffen noch nicht weiter eingebürgert hat."

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[1] According to Odhner, 1928 (Arkiv. f. Zoologi, Vol. 20), *Steringotrema pulchrum* S. J. Johnston 1913 is identical with *Gastris consors* Lühe 1906.

Commenting on Odhner's action, Woodcock (1912) stated that, ". . . this change in name appears to contravene the usually accepted rules," and referring to the family name this author observed that ". . . the name should be Fellodistomidæ as the author (Odhner) himself recognizes." Nicoll (1913) further stated, "It is obvious that the name Steringophorinæ cannot stand but must give place to the earlier Fellodistominæ. The name of the family should consequently be changed to Fellodistomidæ." In a later paper, Nicoll (1915) used the family name Fellodistomidæ without comment.

Poche (1925) attempted to justify Odhner's change of name but his argument appears to be beside the point as will be shown later. Fuhrmann (1928) adopted Odhner's classification and in the subfamily Steringophorinæ included *Steringophorus* Odhner, *Fellodistomum* Stafford, *Rhodotrema* Odhner, *Steringotrema* Odhner (syn. *Pycnadena* Linton), *Didymorchis* Linton, and *Bacciger* Nicoll. It should be pointed out that *Didymorchis* Linton 1910 was preoccupied, and the following year Linton (1911) proposed the name *Pycnadena* for it. There appear to be too many differences between *Steringotrema* and *Pycnadena* to regard them as identical, and Fuhrmann's statement of synonymy is probably an error.

It will be noted that in Odhner's arrangement, Steringophorus is named not only as type of the subfamily but of the family as well and that Steringophorinæ is designated as type subfamily. Poche based his argument on the provision in the rules of nomenclature that the name of a family or subfamily is to be changed when the name of the type genus is changed. It is obvious, however, that the name of the type genus of Nicoll's subfamily Fellodistominæ was not changed in Odhner's arrangement. Instead, another genus was selected as type. The opinion of Professor Ch. W. Stiles was asked concerning the status of Odhner's action and the validity of the subfamily name Steringophorinæ. In a personal communication he makes the following statement, " Steringophorinae is a deliberate renaming of the subfamily Fellodistominae.

" On page 98, Odhner gives a footnote in which he explains why he renamed the subfamily. His explanation shows that he confused two elements, namely, the genus which forms the nomenclatorial type and the genus which he looked upon as the anatomical norm. This is not an uncommon confusing which occurs in systematic zoölogy and is due to the fact that the word " type " is used in so many different senses. According to Odhner, Fellodistomum, the nomenclatorial type of Fellodistominæ, represents a peripheral genus from his point of view, while Steringophorus represents the anatomical norm. This, of course, is a point of view, but in the last analysis, is somewhat subjective and may be changed by a division of the subfamily by some future author.

" The important point is that Fellodistomum is the nomenclatorial type of the first available subfamily name.

" If Odhner's method of nomenclature were applied generally to zoölogy, there would be numerous unnecessary changes in family and subfamily names. On basis of Odhner's statements, Steringophorinæ is subjective synonym of Fellodistominæ. It is subjective rather than objective because it has a different type genus. I would not hesitate an instant in this case, I would use Fellodistominæ."

The analysis and decision of Professor Stiles is so incisive and pertinent that its publication is a valuable contribution to zoölogical literature. It outlines correct procedure and stands in contrast to the confused and irrelevant argument of Poche. Since Fellodistominæ is accepted as the type subfamily of the family to which it belongs, the family name must be Fellodistomidæ. So far as has been determined, the subfamily includes the following genera: *Fellodistomum* Stafford 1904, *Steringophorus* Odhner 1905, *Pycnadena* Linton 1911, *Rhodotrema* Odhner 1911, *Steringotrema* Odhner 1911, *Bacciger* Nicoll 1914, and *Lintonium*, gen. nov.

SUMMARY

Additions are made to the description of *Distomum vibex* Linton. Since *Distomum* is not a valid generic name, and since the species cannot be assigned to any known genus, the new genus *Lintonium* is erected to contain it. The genus belongs to the subfamily Fellodistominæ, Family Fellodistomidæ (Syn. Steringophoridæ).

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THE MANNER OF SPERM ENTRY IN THE STARFISH EGG

ROBERT CHAMBERS

(From the Eli Lilly Research Division, Woods Hole, and Washington Square College, New York University)

In an article published several years ago (Chambers, 1923) I described some morphological aspects of the insemination of the starfish egg. A peculiar feature in this process, the interpretation of which has been adversely criticized (Lillie and Just, 1924; Just, 1929) is the apparently passive and relatively slow travel of the blunt-headed spermatozoön through the jelly which surrounds the egg.

There is a striking contrast between the arrangement of the spermatozoa about freshly inseminated starfish (*Asterias*) and sea-urchin (*Arbacia*) eggs. In *Arbacia* the pointed, narrow-headed sperm quickly pass through the jelly surrounding the eggs and, within a few seconds after insemination, are on the surface of the egg. In *Asterias* the blunt, ovoid sperm penetrate very little into the jelly and collect on its outer border far from the surface of the eggs. By careful observation, one is able to detect a spermatozoön, advancing through the jelly by a peculiar gliding movement to the egg. As described in my previous paper, the moment when the spermatozoön starts to migrate through the jelly, it is seen to be connected by a tenuous filament to a conical elevation on the surface of the egg. The spermatozoön advances as the filament progressively shortens until the head of the spermatozoön finally reaches the cone into which it sinks. From there it travels into the main body of the egg.

Fol, who was among the first to describe the penetration of a spermatozoön into an animal ovum (Fol, 1877) made an extensive study of the process in *Asterias* and *Toxopneustes* (Fol, 1879). In his studies on the starfish he was struck by the peculiar directive movement of the spermatozoön through the jelly to a conical elevation on the surface of the egg and considered the possibility that the progress was due to the retraction of a filament, connecting the spermatozoön with the cone, Fig. 1. He dismissed the idea that the filament is an outgrowth of the spermatozoön, since he observed no diminution in volume of the head. He also suggested that protoplasmic filaments may pre-exist extending from the egg through the jelly and that a sperm, coming into contact with one of these filaments, may be drawn in by a reaction on the part of the egg. Not being able to observe such a filament except as a comparatively short extension of the cone, Fol concluded that the initial

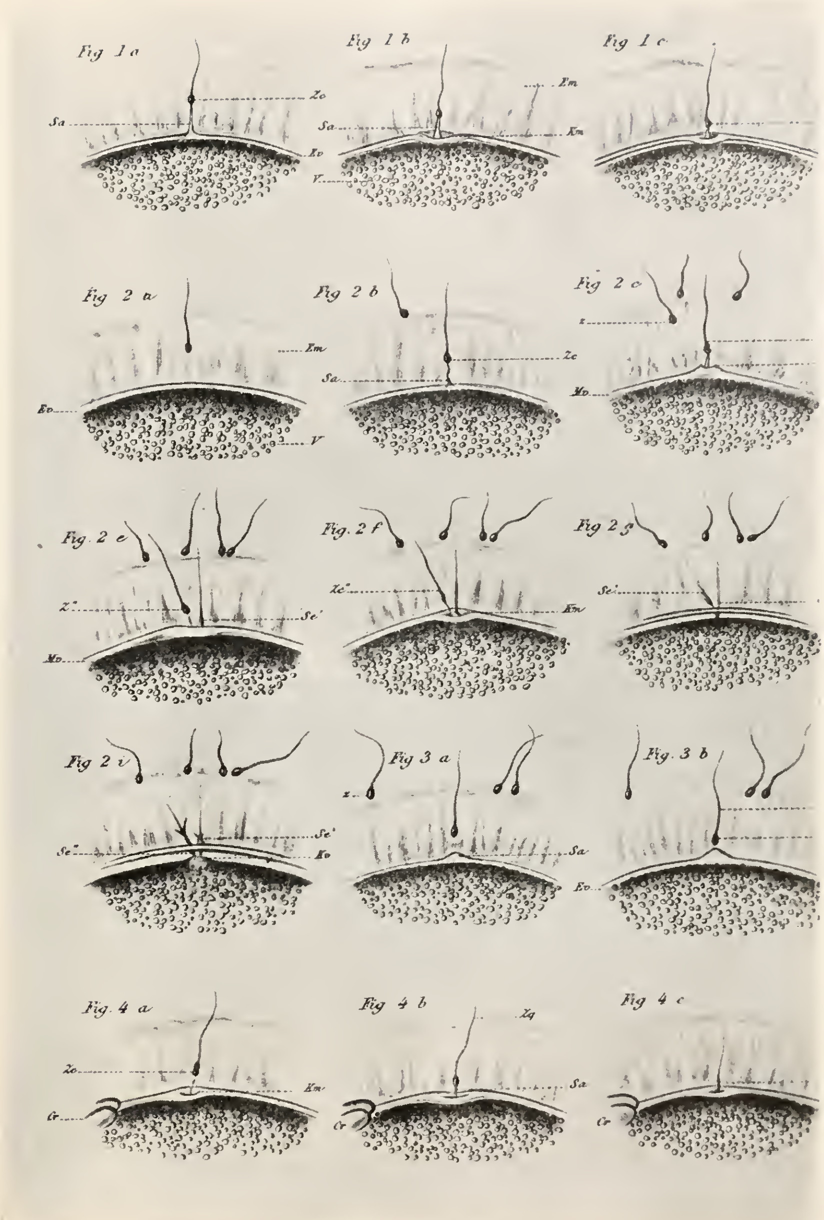
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Fig. 1. Photographic reproduction of part of Plate III from Fol's paper (1879) on *Asterias glacialis*, the drawings of which were from the living egg. In Fig. 1, *a*, *b*, and *c* are three successive phases of the same zoösperm, *zc*. An extension of the entrance cone is at *Sa*. The phase in which the zoösperm entered is omitted here. In Fig. 2, *a*, *b*, *c* (*d* omitted) *e*, *f*, *g*, (*h* omitted) and *i* are seven views of the same objects; in Fig. 2, *b*, *c*, a zoösperm, *zc*, is approaching. In *e* and *f* a second zoösperm, *z″*, is approaching. In *g* and *i* are extensions, *Se′* and *Se″*, of the "cone d'exudation." Fig. 3, *a*, *b*, shows the approach of a zoösperm to an exceptionally large cone. Fig. 4, *a*, *b*, *c*, shows a zoösperm entering near region of polar bodies, *Cr*.

travel of the sperm is due to an attraction exerted by the cone from a distance. A photographic reproduction of a part of Fol's illustrations is shown in Fig. 1.

The results presented in this paper constitute a critical re-examination of the phenomenon and are based upon observations made at different periods every summer since the publication of my original article.

During the summer of 1929 at the Marine Biological Station, Roscoff, France, I was able to confirm the observations of Fol on the species he used, *Asterias glacialis.* Fol's article is remarkable for its wealth of detailed description and should be referred to by any one interested in the subject.

Methods and Material

Observations were made at Woods Hole on the ova of *Asterias rubens,* the common starfish, during all the summer months from June to September. The ova were obtained both by allowing a ripe female to shed the eggs naturally in sea-water and also by removing and cutting up ripe ovaries in bowls of sea-water.

The insemination process was observed with a 3 mm. apochromatic objective in both immature and mature ova at various times before, during and after completed polar-body formation. The temperature of the water in which the inseminations were made varied at different times of the summer (from 15° C. to 20° C.). A preliminary insemination of a sample lot of the eggs was always made under the conditions of the final experiments and only those kept for a study of the normal process when normal fertilization membranes developed within a few minutes on a minimum of 90 per cent of the eggs. For the crucial experiments precautions were taken to make adequate dilutions of the sperm-suspensions in order to procure maximum fertilization with a minimum of sperm present. Heavily inseminated specimens were also studied.

In all the cases in which the penetration of the spermatozoön was observed, the manner of its entry proved to be essentially the same irrespective of variations in temperature, age of eggs or amount of sperm present.

Fol used the following excellent method for observing insemination. He placed a drop of sperm-suspension on the slide of a compressorium on the stage of the microscope and a hanging drop of sea-water containing the eggs on the coverslip of the cap of the compressorium, which was inverted over the slide. After bringing the sperm-suspension into the field of the microscope, he carefully lowered the cap of the compressor until the two drops touched. The eggs, being heavier than the water in which they were suspended, fell through the liquid, while the sperm rose and encountered the eggs under conditions approaching the normal.

The compressorium used by Fol may be dispensed with if a coverslip be mounted on feet of soft clay and the two drops brought together by pressing down on the coverslip. Owing to the fact that the starfish eggs react relatively slowly (15–45 seconds), the sperm can also be mixed with the eggs in a dish. A drop of the mixture is then placed on a slide and covered for observation. With a little practice one is able to bring the eggs into view under an oil immersion objective within 5–10 seconds. Some of my studies were made with the use of the micromanipulator, the sperm-suspension being microinjected into a hanging drop containing the eggs already under view in the microscopic field. With this method the entire sequence of events could be observed from the moment that the sperm arrived in the vicinity of the eggs.

Experiments were also made in which the microneedle was used to operate on the surface of the egg and to seize entering spermatozoa. For this purpose it was essential to have two observers using a demonstration ocular, one observer maintaining the spermatozoön in focus, while the other observer operated the microneedles. I wish to take this opportunity of expressing my appreciation to Dr. G. H. Faulkner of the University of London, who was of the greatest assistance to me in this way.

The time relations of the several steps in the penetration of the spermatozoön vary within certain limits. Spermatozoa taken directly from the testis are sluggish and frequently motionless, but become active when diluted in sea-water. As long as they are actively motile, the spermatozoa of different batches seem to be similar in their behavior toward eggs of one lot. On the other hand, with eggs of different lots and ages, considerable time-variations occur, although the consecutive steps of the insemination process are the same. Immature eggs, as well as eggs which have maturated and have stood for hours in sea-water can be readily inseminated.

In immature eggs the penetration of a spermatozoön does not always cause the vitelline membrane to rise so as to form the fertilization membrane and, if plenty of sperm be present, the sperm will keep on penetrating until the egg is fairly riddled with them. Polyspermy is also the rule for mature eggs aged for three to five hours.

In freshly maturated eggs the peculiar reaction which prevents polyspermy occurs within an average time of 45 seconds and the fertilization membrane rises rapidly. In some batches of eggs the time limit of sperm-penetration may be only 75 seconds, although the usual limit is two minutes.

EXPERIMENTAL

A. Observational Studies

1. The Jelly Around the Starfish Egg

The clear jelly which surrounds the egg swells in sea-water to form a layer approximately 1/6 the diameter of the egg. The outer border of this jelly can be shown by the well-known method of placing the eggs in sea-water containing a suspension of india ink. In accordance with Fol's findings, the jelly appears to be principally a matting of delicate fibrillæ arranged in radial lines. Its density is greatest close to the egg and progressively loosens on approaching its external border. Fol used an ingenious method to demonstrate the radial structure by placing eggs in sea-water containing rod-shaped bacteria. The bacteria implanted themselves in the jelly and always in lines perpendicular to the egg's surface.

In the immature condition the jelly is bounded externally by a thin cellular membrane which breaks up as the jelly swells in the water. When this membrane is present the spermatozoa do not adhere to it. As soon, however, as the membrane disrupts, the spermatozoa readily accumulate in the peripheral meshes of the exposed jelly.

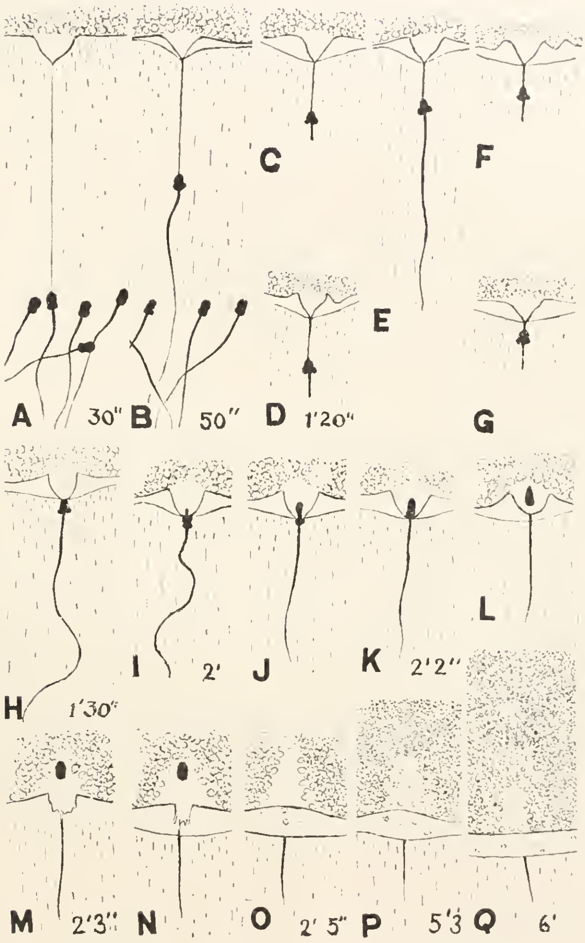
The density of the jelly is such that the starfish spermatozoa with their blunt heads remain entrapped in its outermost zone while their tails continually lash to and fro. On the other hand, the narrow-headed sand-dollar and sea-urchin sperm can work their way quite through the jelly of the starfish egg. Their progress is somewhat impeded the farther they penetrate, but they arrive at the surface of the egg within one or two minutes. This is in striking contrast to the few seconds which it takes them to go through the looser jelly of both sand-dollar and sea-urchin eggs.

The jelly of the starfish egg cannot be removed entirely by mechanically shaking the eggs, although such a procedure is frequently successful for sea-urchin and sand-dollar eggs.

2. Insemination of the Freshly Maturated Egg

In an inseminated preparation of eggs in sea-water a microscopic examination will show the spermatozoa adhering to the outer borders of the sticky egg-jelly. As long as the spermatozoa do not touch the jelly they are as likely to swim away from the egg as towards it.

Fig. 2 (*A–Q*) represents seventeen successive steps in the passage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N
O
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P
5'3
Q
6'

Fig. 2. Seventeen successive steps in the insemination of a starfish egg. For description see text.

of a spermatozoön through the egg-jelly and into the egg until the diminutive sperm-aster becomes appreciable. The drawings were made mostly from observations on one specimen obtained from freshly matured eggs shed naturally in a tank and inseminated with a minimum dilution of spermatozoa to ensure proper insemination. The preparation was brought under observation (with a 3 mm. apochr. objective) within 10 seconds after mixing the eggs with the spermatozoa.

In Fig. 2, *A* several spermatozoa are shown in the outer border of the jelly. When first observed, the head of one of these was already connected by means of a distinctly appreciable but tenuous filament extending through the jelly to a hyaline, conical papilla on the surface of the egg.[1]

Twenty seconds later the spermatozoön had moved about halfway in, Fig. 2, *B*. Its progress was steady and in a straight line, while the tail stretched out motionless behind and only occasionally gave a spasmodic twitch. The fertilization membrane was already to be seen beginning to rise from the cone at the base of the filament. The successive steps in the advance of the spermatozoön to the summit of the cone (Fol's cone d'attraction) are shown in *C* to *H*. While this was occurring, wave-like quivers (see *D* to *G*) passed over the cone and the adjacent surface of the egg. When the spermatozoön reached the summit of the cone, there was an appreciable pause of 30 seconds, after which the sperm-head narrowed at its tip and lengthened out as it slipped through the fertilization membrane to round out again after it has passed into the underlying cone (*I* to *K*). The changes in the shape of the head of the spermatozoön suggest the existence of a pore in the rising membrane through which the filament had previously extended and which is now the means of ingress for the spermatozoön. When once the spermatozoön started to enter, it slipped through rapidly and, within 2–3 seconds, passed definitely into the egg, where its progress (*N–O*) could be followed along an ever-deepening, hyaline pathway caused by a recession of the cytoplasmic granules. As the sperm-head advanced in the egg it became increasingly difficult to see. Within 6 minutes after insemination, the diminutive sperm-aster (*P* and *Q*), became evident at the bottom of the pathway. The path gradually disappeared as granules moved back into it. Usually it is visible for 8 to 10 minutes after insemination.

During the progress of the spermatozoön through the jelly the sperm-tail is relatively inactive. Frequently a spermatozoön moves all the way to the insemination cone without a single twitch of its tail. A pronounced lashing of the tail occurs only during the pause after the spermatozoön has reached the cone, Fig. 2, *H,* and while it is passing through the fertilization membrane, Fig. 2, *I–F.* As long as there is a continuity between the tail and the advancing head within the egg, the tail keeps on feebly lashing. When the connection with the sperm-head is lost, the tail becomes motionless, but can be recognized for a long time (ten to fifteen minutes), extending outward from the fertilization membrane, Fig. 2, *N–Q.*

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[1] Some of the best observations I have made of this phase were with dark-ground illumination.

The fertilization membrane usually becomes evident in the region of the cone before the spermatozoön has migrated halfway through the jelly, Fig. 2, *B.* Its complete elevation over the egg occurs within 5 to 20 seconds later.

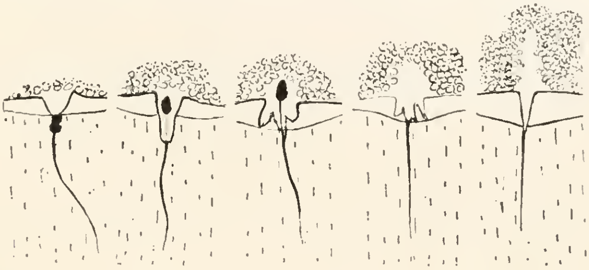

FIG. 3. Progressive changes in the form of an exudation cone.

The conversion of the entrance cone into the exudation cone (Fol's cone d'exudation) takes place after the spermatozoön has passed into the egg. Ever-changing, flame-like processes develop on the cone, Fig. 2, *M, N,* which finally withdraw and the cone disappears, frequently leaving behind minute globules, Fig. 2, *O–Q,* which become dispersed in the space between the fertilization membrane and the egg. A variation of the exudation cone is shown in Fig. 3.

In over-inseminated eggs several spermatozoa may become attached, Fig. 4, *A,* each to the tip of a filament extending from the egg. Al-

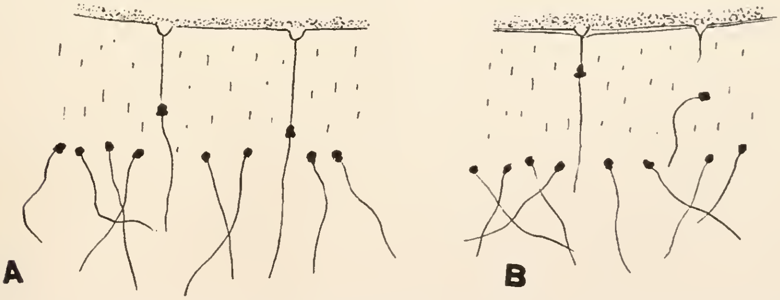

FIG. 4, *A.* Two spermatozoa migrating together into an over-inseminated egg. *B.* One lost its attachment and was discarded, while the other successfully entered the egg.

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though these spermatozoa begin to move through the jelly, there is a tendency for only the most advanced one to reach and penetrate the egg. The others, before reaching the egg, tend at one time or another to lose connection with their filaments. Such released spermatozoa, after a spasmodic twitch or two, remain permanently motionless, Fig. 3, *B,* in the jelly. The filaments which have lost their spermatozoa are quickly withdrawn and, together with their cones, soon sink into the egg.

The filaments, extending from a cone to a spermatozoön, are usually at right angles to the egg's surface. That this is not always the case is shown in Fig. 5, where two convergent filaments are shown. This argues against the pre-existence of definite radial canals in the egg-jelly through which the spermatozoa might be supposed to move.

The shape of the head of the spermatozoön, as already commented upon by Fol, occasionally changes considerably as the head moves through the jelly. The change seems to be due mainly to a bulging of the neck-piece on one or both sides of the head, Fig. 6, *A, B* (*cf.* Fig. 5). In Fig. 6, *B* are three sketches of a single spermatozoön, at intervals of 20, 40 and 60 seconds after insemination. The impression that the head of the spermatozoön is bent to one side may be due to the distorted shape of the neck-piece. Occasionally, a spermatozoön appears to be carried through the jelly with the base of its tail at right angles to the attachment of the insemination filament, while the rest of the tail is curved so as to trail behind.

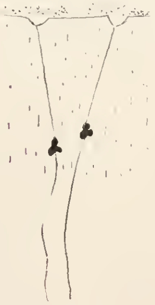

Fig. 5

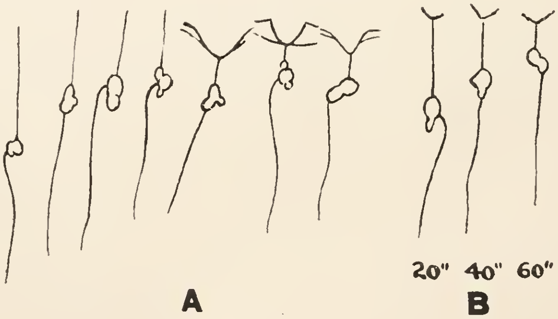

Fig. 6

Fig. 5. Two spermatozoa attached to insemination filaments which are convergent and not radial as usual.

Fig. 6. *A.* Sketches to show variations in shape of the heads of spermatozoa migrating through the egg-jelly. *B.* Changes in shape of the head of one spermatozoön at intervals of 20, 40, and 60 seconds.

Figures 7–10 represent variations. Fig. 7 shows a sperm-head which was unusual in performing active, wriggling movements for fully one minute after having penetrated the egg while the tail hung motionless outside. During these movements the sperm-head left the usual hyaline pathway and could be seen jostling and pushing aside the cytoplasmic granules encountered.

Fig. 8 shows a spermatozoön whose head, after passing through the fertilization membrane, broke away from its neck-piece which was left outside with the tail.

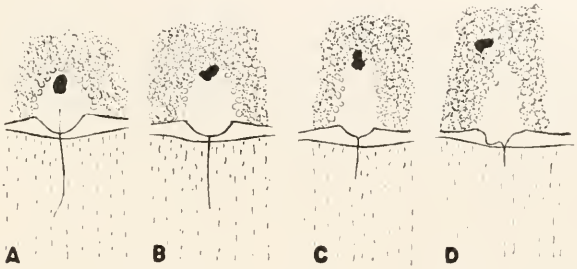

Fig. 7

Fig. 7. Four successive steps in the progress of an unusually active sperm-head after it had penetrated an egg.

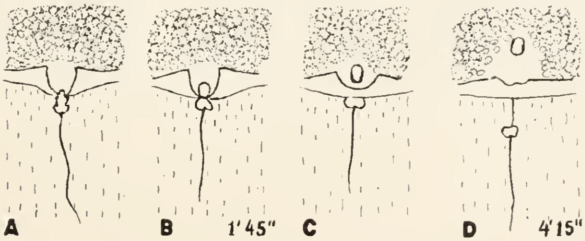

Fig. 8

Fig. 8. A spermatozoön which on entering an egg left its neck-piece outside the fertilization membrane.

Figs. 9 and 10 show the reactions of late arriving spermatozoa. Fig. 9 shows a spermatozoön which succeeded in passing through an already lifted fertilization membrane. During the process the cone changed shape and flattened out, while the fertilization membrane became appreciably indented. In Fig. 10 the spermatozoön reached the cone, *A–C,* but failed to enter. The fertilization membrane wrinkled and the cone formed accessory elevations, *D–F,* but, when the cone finally withdrew from the membrane, the spermatozoön was left outside. The head of the spermatozoön then sprang back for a short distance where it remained motionless and attached to the membrane by a slender thread, *G,* nine minutes after insemination.

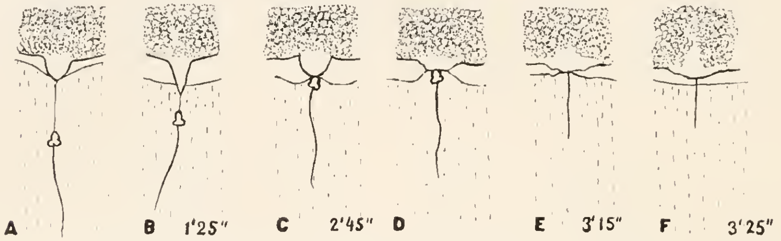

Fig. 9. Delayed entry of a spermatozoön through a fertilization membrane formed by the penetration into the egg of another spermatozoön not shown in the figure.

3. The Origin of the Insemination Filament

The insemination filament is so fine that it is practically invisible except when the cone at one end and the sperm-head at the other end are brought simultaneously into focus. Considerable practice is required to detect the sperm at the moment when it is beginning to migrate into the jelly. In the outer border of the jelly among several spermatozoa whose heads are moving to and fro while their tails lash about, one's attention becomes attracted to a sperm-head which has ceased its side-to-side movements and, instead, is moving steadily and in a straight line into the depths of the jelly. By looking along the direction of its movement, a cone on the egg's surface becomes apparent and, between the cone and the sperm, is to be seen the delicate, tenuous insemination filament. In fresh maturing eggs I have never been able to see the cone without also seeing the advancing sperm and the filament connecting the two. The formation of the filament is apparently too rapid. In immature eggs the cone is relatively much larger and as already described (Chambers, 1923) I have several times observed a tapering extension grow out from it until contact is made with a sperm, whereupon the extending portion retracts and draws the sperm in with it.

In mature eggs which have been standing in sea-water for 2 to 4 hours there is frequently a greater response to multiple cone formation than in fresh, maturing eggs and consequently the chances are better to catch the initial stages. Eggs, 3 hours old, were placed in a shallow hanging drop in a moist chamber and, after being brought under observation, a suspension of sperm was blown into one side of the field by means of a micro-pipette. The spermatozoa quickly spread in the interstices between the eggs and several became attached to the outer border of the jelly of the egg in view. Within 10 seconds a number of minute, conical, blister-like elevations developed on the egg's surface opposite the sperm. A delicate membrane appeared as if it were being lifted off the egg's surface by the rising cones. A few of the hyaline cones increased in size and, during the several succeeding seconds, there was no sign of any connection between them and the sperm lying on the periphery of the jelly. One cone increased appreciably in size and suddenly, within an instant, a distinct line could be seen connecting its tip with the head of a spermatozoön. The other spermatozoa remained on the surface of the jelly while the spermatozoön in question began to migrate inward. While this was occurring, the rounded surface of the cone tapered more and more and the ever-shortening filament became appreciably thicker.

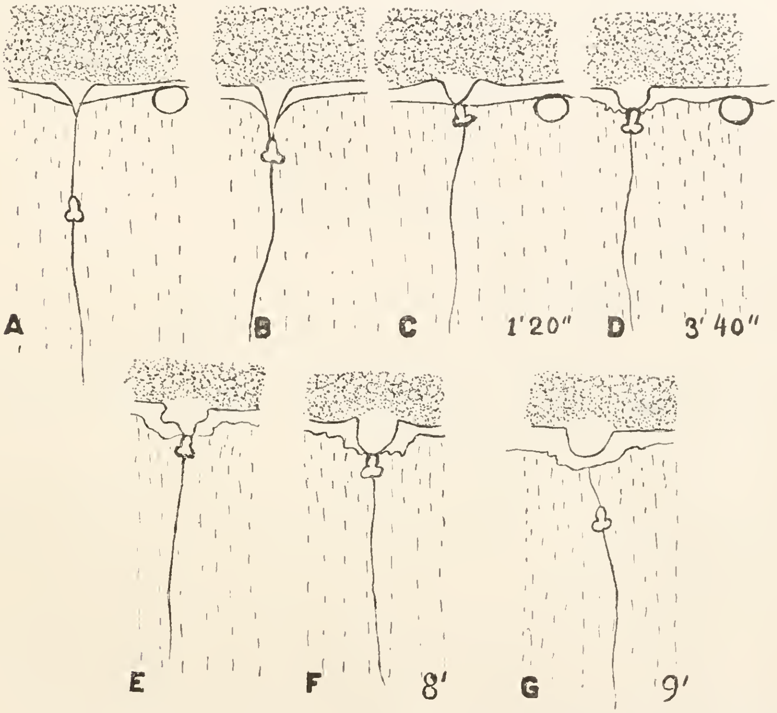

FIG. 10. Attempted penetration of a delayed spermatozoön which was finally discarded.

A curious phenomenon which may be of significance is the fact that, in the majority of cases, the insemination filament always connects with a spermatozoön. Because of this one is almost inclined to believe in a specific attraction such as Fol suggested. I may cite, for example, a case in which about 30–50 spermatozoa were blown on the surface of an egg. Most of the spermatozoa immediately became attached to a restricted region on the outer border of the jelly. One, however, wandered off a short distance and suddenly a cone appeared with a tenuous filament extending to the spermatozoön diagonally through the jelly. The filament then retracted with the spermatozoön on its tip and insemination resulted.

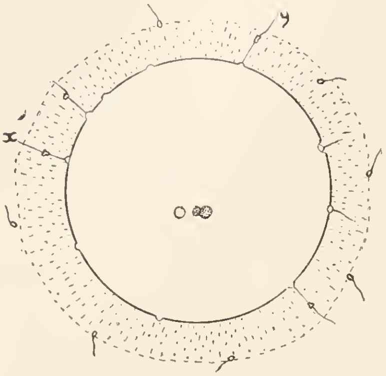

FIG. 11

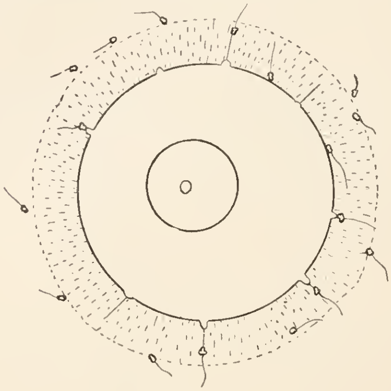

FIG. 12

FIG. 11. Polyspermy in an egg 5 hours old. The egg nucleus and two polar bodies show prominently in the middle of the figure. Sperm at *x*, although more advanced, entered later than sperm at *y*.

FIG. 12. Polyspermy in an immature egg.

4. Insemination of Immature and of Aged Eggs

Eggs aged by standing in sea-water lose their protective reaction against polyspermy. Fig. 11 represents an egg which was inseminated after it had been standing in sea-water for five hours, which is over four hours longer than is usual for normal fertilization. Within one minute numerous cones formed on the egg. The figure shows the egg with six attached spermatozoa, all of which were taken in. Owing to the rapidity of the procedure and the variations in the angles of direction which the filaments take, it was impossible to ascertain whether or not the cones in the figure which show no filaments did in reality possess filaments with spermatozoa attached to them.

There is often a lack of uniformity in the sequence of the sperm entries. In Fig. 11 the spermatozoön at *x* was in advance of its neighbor at *y*. In spite of this, spermatozoön *y* entered before *x*.

One egg, two hours after maturation, formed two cones with insemination filaments at an interval of two minutes. Both successfully drew in their spermatozoa. One minute later another cone and filament developed. Its spermatozoön began to be drawn in, but the rising fertilization membrane had appreciably formed and the spermatozoön was discarded.

Another egg, 5 hours old, formed a large number of cones so close together that, as they enlarged, they became more or less confluent and spermatozoa kept migrating into them in large numbers, Fig. 13.

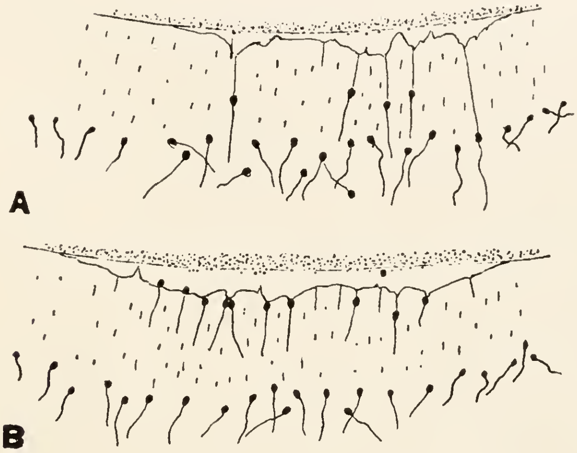

Fig. 13. Excessive over-insemination with formation of confluent cones.

The lack of a protective reaction against polyspermy in old, mature eggs obtains also for immature eggs. This is shown in Fig. 12. The entrance cones which form on the immature egg are distinctly larger than those of the mature egg.

As the sperm passes into an immature egg no hyaline pathway is formed such as occurs in the mature egg. The spermatozoön is quickly lost to view among the cytoplasmic granules and no aster ever develops. Also the exudation cone which forms at the site of the disappearing entrance cone usually develops into a strikingly large prominence with elongated flame-like processes. A membrane similar to the fertilization membrane of mature eggs forms about an immature egg upon insemination. In fresh, immature eggs the membrane seldom rises. It simply toughens as can be demonstrated by the microneedle. In old eggs, which remain immature by maintaining an intact germinal vesicle, the membrane frequently lifts off upon insemination.

5. Time Relationships in the Insemination Process

The time relations of events in the insemination process are shown in the accompanying table, in which records are given on observations of a number of individual eggs.

After the eggs and sperm are mixed there is always an appreciable time of 20 to 35 seconds before the first spermatozoön begins definitely to migrate into the jelly. The average time to pass through the jelly is 60 seconds. The spermatozoön remains on the surface of the entrance cone for about 25 seconds, after which it rapidly penetrates the cone and passes into the interior of the egg. The diminutive spermaster becomes appreciable within 5 to 6 minutes after insemination. Within certain limits the sequence of events for fresh, maturing eggs is fairly uniform. The greater variability in old eggs may be due to the fact that aging eggs permit polyspermy and hence the data probably include records on the penetration of late as well as early arrivals.

A comparison of my data with those recently published by Just (1929) and included in the table shows agreement in one essential point, *i.e.*, in the average time taken after insemination for the sperm to enter the cone, viz., 120 seconds. The disagreement lies in the time taken for the sperm to arrive on the cone. Although Just states that he made his observations both on living and fixed eggs, careful perusal of his paper suggests that he depended more on data obtained from fixed and sectioned material than from observations on the living egg. According to my observations, the spermatozoa were never observed to reach the surface of the egg in less than 45 seconds. I cannot explain Just's statement that this occurs within 5 seconds except on the assumption that throwing the eggs into a fixative might possibly induce a sudden contraction of materials so as to bring the sperm on the cone before the fixing agent had time to exert its preservative action.

B. Microdissection Studies

6. Physical Properties of the Cone and of the Insemination Filament

The entrance cone possesses a surprising stiffness somewhat at variance with the impression it gives to the eye from its ever-changing contour.

A cone, Fig. 14, *A*, into which a spermatozoön had just entered, was pushed inwards by means of the tip of a microneedle bearing down on the fertilization membrane, *B*. The relative stiffness of the cone was indicated by the fact that the general contour of the egg about the cone was carried in while the cone persisted in its original form within the

TABLE

Time Relations in the Process of Insemination in Asterias rubens

	Condition of Eggs	Duration of Process	Sperm Added	Spermatozoön				Fertilization Membrane	
				On Filament		Arrived on Cone	Within Cone	Started	Completed
				At Periphery of Jelly	Half Way through Jelly				
My Data	Polar bodies being formed	Longest recorded	0″	45″	60″	100″	135″	45″	60″
		Shortest recorded	0″	15″	30″	55″	80″	30″	35″
		Average (20 observations)	0″	30″	50″	90″	115″	40″	50″
	Aged		0″	variable		60–180″	90–210″		
	Immature		0″	variable		60–180″	120–240″		
Just's Data	Both fresh and fixed		0″			5″	120″	45″	

resulting recess. The microneedle was then passed through the fertilization membrane and the surface of the rounded cone was seized and deformed by pulling, *C*. After removal of the needle, the dragged-out part of the cone slowly and gradually withdrew, *D–E*.

In another case the fertilization membrane was first removed by tearing and the cone pulled out into a long tapering strand. While held in this position, the strand became lumpy as if it were breaking into beads. The contour of the cone at its base kept changing, while the lumpiness of the strand progressively disappeared and reappeared. Finally the strand broke into beads. The basal position of the strand, thus freed from the needle, gradually sank into the cone, which ultimately flattened out and disappeared. This phenomenon is similar to what occasionally occurs when the lifting of a fertilization membrane, due to insemination elsewhere on the egg, drags out a retracting filament attached to a spermatozoön outside the membrane.

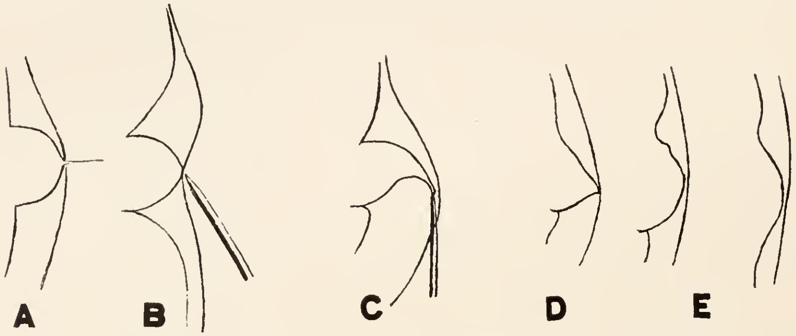

Fig. 14. Micromanipulation of an entrance cone. For description see text.

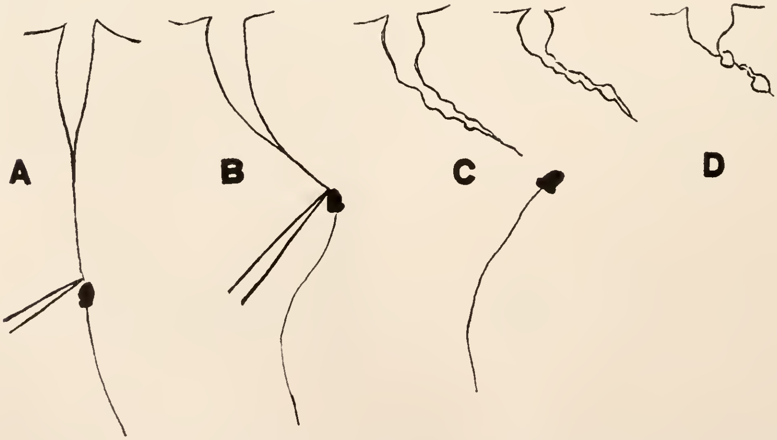

Fig. 15. Effect of removing spermatozoön from the insemination filament.

With a microneedle a spermatozoön was removed from its filament while the sperm was moving through the jelly, Fig. 15. The tip of the needle was raised and moved against the spermatozoön, *A*. In the process the cone became stretched as the filament was pushed to one side, *B*. Eventually the spermatozoön became dislodged, *C*, whereupon the filament retracted and beaded, *D*, while the freed spermatozoön remained motionless in the jelly. In other cases I have tried without success to separate the filament from its cone by manipulating the needle-tip where the filament joins the cone. The filament continues retracting and the spermatozoön moves steadily to the cone except when the operation becomes so brutal as to disrupt the cone.

7. The Effect of Removing the Vitelline Membrane before Insemination

I have already described the fertilization of eggs previously deprived of their vitelline membranes, (Chambers, 1923). The jelly adheres to the membrane which in its turn is closely adherent to the egg. While tearing the membrane the egg is usually injured. Occasionally, however, one is able to insert a fine needle under the membrane, Fig. 16, and lift it off while delicate strands of protoplasm which appear, stretch and break. The following experiment indicates that this membrane is the same structure which lifts off as the fertilization membrane. The membrane was partially torn from the surface of an egg which was then inseminated, Fig. 17. A spermatozoön happened to find its way into the space under the torn membrane, *A*, while the egg was fertilized by another spermatozoön in a region not shown in the figure. The lifting of the fertilization membrane spread over the egg until it reached the torn region, where the presence of the horizontally stationed spermatozoön

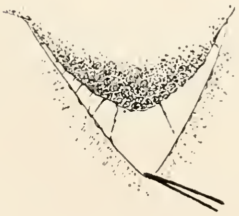

Fig. 16

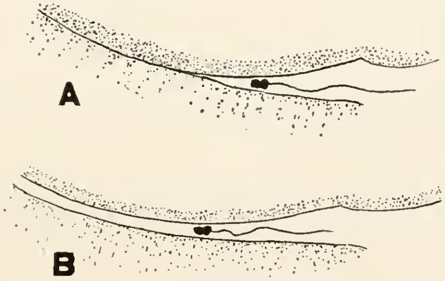

Fig. 17

Fig. 16. Operation of tearing the vitelline membrane of an unfertilized egg.
Fig. 17. An egg inseminated after partially tearing off the vitelline membrane. *A*. Spermatozoön lying in space between vitelline membrane and egg. *B*. Fertilization membrane lifted owing to insemination by a spermatozoön not shown in figure.

showed that the fertilization membrane was identical with the membrane which previously had been torn, *B*. The spermatozoön in the figure advanced somewhat within the space between the egg and the membrane.

Fig. 18 shows the way in which the jelly can be removed from an unfertilized mature egg. After tearing the jelly, the exposed part of the egg is seized with one needle while the jelly at the other end of the egg is caught by a second needle. By gentle manipulation, the egg can be drawn completely out of its jelly. Such an egg at the outset is very sticky. However, by rolling it about, the adhesiveness diminishes and the egg rounds up and cannot be distinguished from untreated eggs except for the lack of an investing jelly.

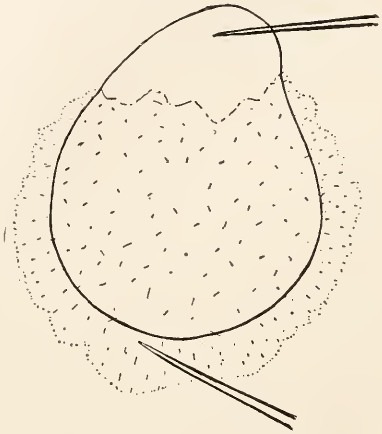

Fig. 18. Method of removing an egg from its investing vitelline membrane jelly.

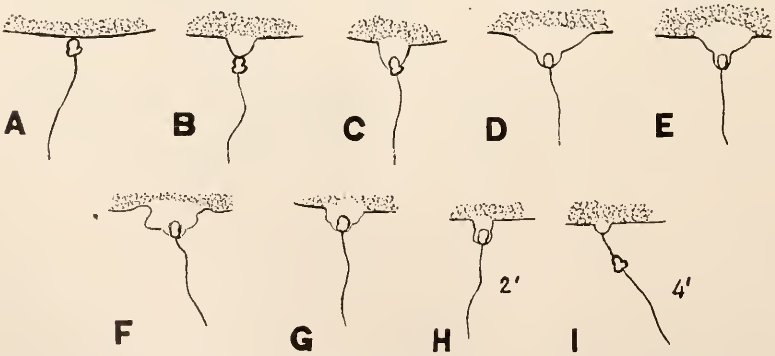

Fig. 19. Several steps in an unsuccessful insemination of a naked egg.

Fig. 19 represents an unsuccessful attempt at fertilizing a naked starfish egg. An entrance cone developed at the spot where a spermatozoön touched it, *A, B*. The head of the spermatozoön was engulfed by the cone, *C* and *D*. However, the sperm-head did not move inward, *E*. Instead, the cone spread out at its base, became irregular, *F*, and then diminished in size, *G* and *H*. Finally the spermatozoön was expelled, *I*, four minutes after it had arrived on the surface of the egg.

Fig. 20 represents the stages of a successful sperm entry in another naked egg. The entrance cone formed as before, *A, B*. It engulfed the sperm-head and then receded as the sperm-head rapidly moved inward along an ever-deepening hyaline pathway within the egg, *C, D* and *E*. The sperm-head produced a typical sperm-aster, *F*, and the egg segmented in a normal manner.

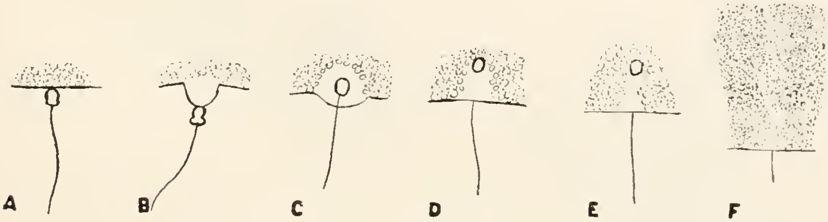

FIG. 20. Several steps in the successful insemination of a naked egg.

The striking features which are brought out in the behavior of the naked egg are as follows: First, the spermatozoön touches the surface of the egg before there is any evidence of a cone. Second, a cone forms after the sperm is in contact with the egg. Third, the cone forms no filamentous process such as is seen when a mass of jelly in-

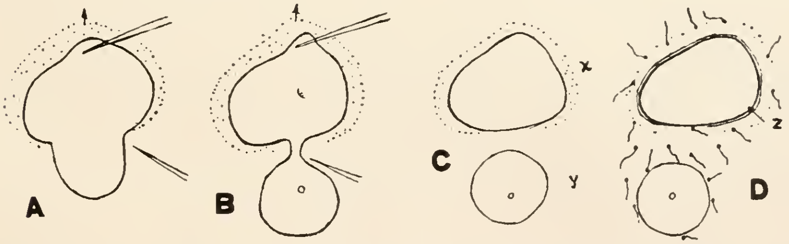

FIG. 21. Production of an endoplasmic exovate by cutting a gash in the cortex of an unfertilized egg and causing the interior to flow out. Ectoplasmic remnant, *x*, is fertilizable. Endoplasmic sphere, *y*, is unfertilizable. For description see text.

tervenes between the cone and the spermatozoön. Fourth, no fertilization membrane whatever is produced.

The insemination of these naked eggs also bears on the question of the existence of a specific attraction of the egg to spermatozoa. Spermatozoa frequently swim up to a naked, unfertilized egg, wander along its surface and then swim away. Apparently the formation of an entrance cone is dependent on something more than the mere presence of a spermatozoön on its surface.

8. Insemination of Squashed Eggs

These experiments show the behavior, toward spermatozoa, of the egg-cortex as contrasted with that of the extruded interior.

Fig. 21 represents the artificial production of an endoplasmic exovate and the behavior of the isolated exovate and of the ectoplasmic remnant to insemination. A deep gash was first made with a needle in one side of an unfertilized, mature starfish egg. With a second needle the other side of the egg was seized and pulled to the shallow edge of a hanging drop, *A*. The interior of the egg flowed out at the spot where the gash was made. The fluid exovate rounded up as its connection with the more solid, cortical remnant of the egg became constricted. By gentle manipulation, *B*, the neck pinched off so that the egg was thus divided, *C*, into an ectoplasmic remnant still maintaining its jelly investment, *x*, and a naked endoplasmic sphere, *y*. As already described (Chambers, 1923), the endoplasmic spheres are unfertilizable. On the other hand, the ectoplasmic remnant is readily fertilizable and may develop into a swimming larva. The difference in behavior of the two pieces when inseminated is shown in *D*. The ectoplasmic remnant produced an entrance cone with its filament and the attached spermatozoön readily entered, *z*, in *D*, and was followed by the lifting of a typical, though collapsed, fertilization membrane. The endoplasmic sphere showed no reaction to the presence of the spermatozoa. Some hit it head on, others wandered over its surface, sometimes remaining motionless for a few seconds, only to swim away. No cones formed on the sphere and no spermatozoön was ever observed to enter. In a previous communication (Chambers, 1921) I stated that the endoplasmic spheres never segment, although I assumed that spermatozoa may enter. This assumption was based on the sections of several endoplasmic spheres which contained numerous small chromatic bodies which I took to be unaltered sperm-heads. In the light of more recent results I reexamined the slides containing these sections and found that the chromatic bodies are far too small to be sperm-heads; they also differ in being rod-shaped and are probably bacterial organisms. They certainly are not spermatozoa. All the other endoplasmic spheres (eighteen in all) which were sectioned and stained showed no bodies even remotely resembling sperm-heads, although they had been heavily inseminated before fixing.

Fig. 22, *A* shows an egg which was torn and squashed. The original cortex was maintained on the part still covered by the jelly. Upon the addition of spermatozoa, cones formed on the original cortex at *x* and *y*. Two minutes later, *B*, the sperm at *x*, had entered while the two sperm at *y* were discarded. Note that the fertilization membrane formed only on the original cortex.

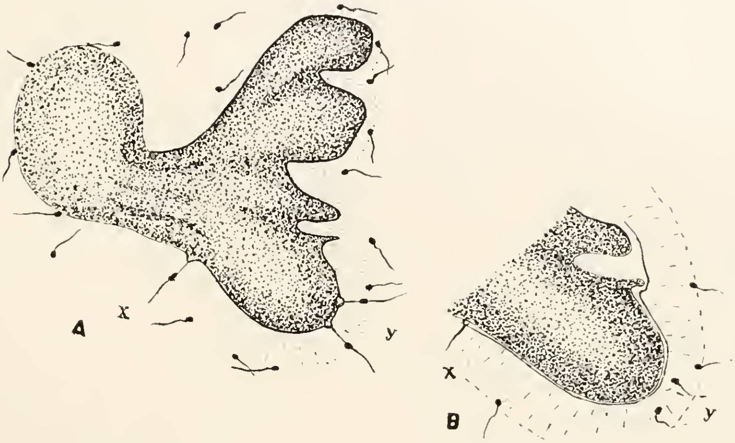

FIG. 22. Insemination of a torn and squashed egg. For description see text.

DISCUSSION

Filamentous structures have been known to develop on the surface of Echinoderm ova. Some of them are delicate, wavy, cylindrical bodies which often appear when the eggs are placed under abnormal conditions of pressure, temperature, hypertonicity of their environment, etc. They are probably degeneration phenomena.

Other filamentous structures of quite a different sort have been noted on eggs after exposure to spermatozoa. Such are the structures described by Seifriz (1926) and Hobson (1927), which are identical with the flame-like processes which Fol long ago described as growing out from the " cones d'exudation " at the site of sperm-penetration. In immature eggs these flame-like processes attain considerable lengths. They slowly change in shape and size although Seifriz found them to be extraordinarily stiff when manipulated with microneedles. The insemination filaments described in this paper resemble the flame-like processes of the exudation cones except that they are extremely tenuous and usually are single instead of multiple. They also possess a stiffness which is quite at variance with the limp, filamentous outgrowths on degenerating eggs.

The results described in this paper indicate that the insemination filaments of mature eggs develop with extraordinary rapidity, but when they retract the process is a gradual one. Because of this, it has been impossible to determine directly whether the insemination filament is an outgrowth from the sperm-head to the cone or whether it emanates from the cone itself. Fol argues against the former possibility, because there is no apparent decrease in volume of the sperm-head. Another case in point is the relatively weak attachment of the filament to the sperm-head, for, whenever the filament is broken, either mechanically or spontaneously (e.g., in the case of incomplete polyspermy), the separation occurs at the head of the sperm and not at the cone. The main argument in favor of the filament being an outgrowth of the entrance cone is that it has been actually observed to develop from the cone in immature and in old, mature eggs in which all the other steps of the insemination process are identical with those of freshly matured eggs. Occasionally an abortive filament has been observed to arise from a cone without encountering a spermatozoön, and later to recede.

The development of the typical insemination filament appears to be a peculiar adaptation to the presence of the radially structured jelly about the eggs, because, when the jelly is completely removed, no filaments develop and insemination occurs by the elevation of an ovoid cone which engulfs the spermatozoön.

An extraordinary feature in the insemination process of the starfish egg is the apparently passive rôle which the spermatozoön plays in its migration through the jelly to the entrance cone. All the evidence indicates that the movement of the spermatozoön is due to the progressive shortening of the insemination filament. In this regard it is significant that occasionally the connection of the filament with the head of the spermatozoön is at such an angle that the spermatozoön moves as if it were actually being dragged backward to the cone. The spermatozoön in such a position could hardly be moving under its own motive power.

The main evidence for concluding that the insemination process described in this paper is normal, is the fact that the fertilization membrane always first rises over the cone at the base of the filament to which the approaching spermatozoön is attached and its elevation then spreads progressively from this site over the entire surface of the egg.

In the presence of too many sperm an egg frequently responds by developing more than one filament with the result that several spermatozoa begin to migrate through the jelly. As the eggs age there is an increased production of filaments. The successful penetration into the egg of one spermatozoön and the failure of another to do so is conditioned by a definite time relation. It is possible for all of several spermatozoa to penetrate the egg if they begin migrating through the jelly within a few seconds of one another. Their success in entering the egg bears no relation to their distance from one another on the surface of the egg but to the time when the filaments begin to draw them in. In freshly matured eggs polyspermy tends to be prevented because of the paucity of insemination filaments. If, out of several migrating inward, one spermatozoön is sufficiently ahead of the others, polyspermy may be prevented by a gradual attenuation of the delayed filaments which finally break loose from the spermatozoa attached to them. Sometimes a delayed filament does not lose its spermatozoön, but continues retracting until the spermatozoön arrives on the cone. The spermatozoön, however, fails to enter the cone because of the elevating fertilization membrane which has already begun to spread from the region of another more successfully functioning cone. In such a case the spermatozoön is definitely discarded by a peculiar process which Just evidently saw when he described a spermatozoön being "pushed off from the egg, a delicate strand connecting the tip with the apex of the cone."

Just (1929) claims that filaments which are formed as a response to insemination occur only on abnormal ova and are exaggerated entrance cones. The only observation which he records of a strand connecting the sperm with the cone is one which he states occurred when the sperm was "pushed off from the egg." Such a case I have also frequently observed on abnormal eggs. My crucial observations of the true insemination filament were on fresh maturing eggs, from lots of which over 95 per cent segmented and developed normally. Fixed material is not suitable for a study of the movement of spermatozoa to the surface of the egg. Our difference of opinion on living eggs is one of observation, the methods we both used being presumably the same.

Quoting from Just, the "spermatozoa rush toward the jelly hull; of these, one, rapidly moving through it, reached the egg within 5 seconds." Although this rapidity of the movement is greater than any which I have observed, it is to be noted that Just admits the passage, through the jelly, of only one out of many; the others remain outside.

I have shown the phenomenon to several competent cytologists at Woods Hole during the past summer. They agreed with me in the observation that the one migrating spermatozoön, during its passage through the jelly to the egg, is connected by an ever-shortening, straight filament to the entrance cone into which the head of the sperm finally disappeared. Moreover, the elevation of the fertilization membrane was observed to start over the base of this particular cone.

SUMMARY

1. Evidence is given to indicate that the formation of insemination filaments is the normal procedure of fecundation in the starfish egg. These filaments extend from the egg's surface to the spermatozoa lying on the outer borders of the jelly surrounding the egg.

2. The spermatozoön on the end of an insemination filament moves to the egg through the jelly by no apparent motive power of its own. This movement is accompanied by a progressive shortening and thickening of the filament.

3. The fertilization membrane begins to rise off the cone by the time the spermatozoön has migrated about halfway through the jelly. The elevation of the membrane spreads from this region.

4. The filament is a peculiar adaptation to the presence of the relatively dense jelly surrounding the egg and to the inability of the blunt-headed spermatozoa to reach the egg. In the absence of the jelly only an ovoid entrance cone develops to receive the spermatozoön.

5. Polyspermy can be prevented by the breaking loose of supernumerary insemination filaments from their attached spermatozoa. The discarded spermatozoa remain motionless in the jelly while the filaments are completely withdrawn.

6. There is a definite relation between the time that two or more spermatozoa become attached to insemination filaments and the success of one or all to enter the egg. This bears no relation to the distance of their places of attachment on the surface of the egg but to the time when the filaments begin to retract.

7. The original cortex is the only part of the starfish egg which responds to insemination. Endoplasmic exovates do not become inseminated.

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